

Development of Novel Model Systems to Assess Replication of Patient Derived Hepatitis C Virus in Tissue Culture

By

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Dedication

This work is dedicated to my wonderful and
loving parents and family.

Declaration

I declare that all the work in this thesis is entirely my own,
unless otherwise indicated.

Acknowledgement

Working on this project has been a significant academic challenge and without the invaluable support of my supervisor Professor Graham R Foster this would have been difficult to achieve. He has been a constant motivational force behind this and I will be deeply indebted to the support and patience he has shown me throughout these years. I am grateful for his expert guidance and the ability to make time for me despite his many other academic and professional commitments. I am also grateful to my second supervisor Professor Ian Sanderson for his interest in my work.

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Abstract

1. Introduction

The development of novel therapies for chronic hepatitis C virus (HCV) infection could potentially be streamlined by using patient-derived model systems in which drugs can be tested on patient derived viral strains prior to embarking upon clinical trials. Such models have been hampered by difficulties in maintaining primary human hepatocytes (PHHs) from infected individuals in culture and by the lack of a replication model system for patient derived virus. Most *in vitro* models to date do not comprise individual patient virus from all strains of HCV and hence novel antivirals cannot be tested on them. The aim of this research was to develop novel models of replication of HCV that will allow new drugs to be tested against all viral genotypes. We have examined two models - an *ex vivo* liver biopsy model and an HCV monocyte-hepatocyte fusion model.

2. Methods

Liver biopsy fragments from patients with chronic HCV infection were studied to determine whether or not they could be used as a replication model. Biopsy fragments were incubated with DMEM alone or with antiviral drugs for 24h and 48h and viral replication assayed by RT-PCR for HCV RNA. Similar experiments were done with isolated primary human hepatocytes (PHHs) from patients with chronic HCV infection which were then compared with liver biopsy fragments. Various methods were used to extend the longevity of

PHHs. In later experiments we examined a novel model in which CD14 positive monocytes derived from patients with active HCV infection were fused with different liver cell lines in order to observe HCV replication. In this later model viral replication was observed and drug validation experiments were performed examining the effect of telaprevir on infected hepatocytes from genotype 1 and genotype 3 patients.

3. Results

Viral replication was observed in both models. The mean viral RNA concentration in biopsies from patients with genotype 1 HCV was significantly lower after 24 hours' incubation with telaprevir compared to unsupplemented medium ($P=0.0015$). Telaprevir did not have an effect on HCV RNA levels in biopsies from patients with genotypes 2 or 3 HCV. Viability of PHHs was reduced compared to intact biopsy fragments.

In the HCV-replication fusion model, HCV expression was higher in fused cells compared to unfused monocytes ($p=0.0007$). Optimal fusion conditions were achieved by using monocytes and Huh 7.5s in a 1:1 ratio and using 3 minutes incubation in PEG with pH of 7. HCV expression was highest during the first 7 days in fused cells and there was a significant decline in HCV RNA during subsequent days ($p=0.002$). Viral protein production was observed in fused cells by indirect immunofluorescence, which confirmed viral replication. HCV was successfully transferred to Huh7.5 cells using the monocyte 'capture-fusion' approach. Telaprevir showed greater antiviral efficacy in fused/infected cells with genotype 1 compared to those with genotype 3 strain of HCV. Interferon's activity was compromised by transfecting Huh 7.5

cells with PIV5 plasmid. This however did not increase HCV expression in these cells after fusion with CD14 positive monocytes.

4. Conclusion

Fragments of tissue from human liver biopsies can be maintained in tissue culture for at least 24 hours. Our data demonstrates the principle that antiviral agents can be tested *ex vivo* using surplus liver biopsy material and that the results reflect the findings of clinical studies in that telaprevir has efficacy in genotype 1 but not genotype 2 or 3 HCV. Moreover, monocytes may act as sanctuary sites for HCV virions and could be used to transfer virus to hepatocytes *in vitro*. The HCV fusion model can be used to test drug sensitivities for different genotypes as well as individual patient sensitivity to antiviral agents. Further analysis of the clinical utility of these model systems is indicated.

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Abbreviations

2'5' OAS	2'5' Oligoadenylate Synthetases
3'UTR	3' Untranslated Region
5'UTR	5' Untranslated Region
ALT	Alanine Aminotransferase
AST	Aspartate Aminotransferase
ALP	Alkaline phosphatase
APC	Antigen Presenting Cell
ARFP	Alternative Open Reading Frame Protein
BSA	Bovine Serum Albumin
C	Core
CD14 cells	Monocytes Separated by CD14 Microbeads
CHC	Chronic Hepatitis C
CLDN1	Claudin 1 Receptor
cDNA	Complementary DNA
DAPI	4',6-diamidino-2-phenylindole
DC	Dendritic Cell

DEPC	Diethylpyrocarbonate
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic Acid
dsRNA	Double Stranded Ribonucleic Acid
E1	Envelope Protein 1
E2	Envelope Protein 2
EHM	Extrahepatic Manifestation
ER	Endoplasmic Reticulum
EVR	Early Virological Response
FDA	Food And Drug Administration
FITC	Flu- Orescein Isothiocyanate
GAF	Gamma Activated Factor
GGT	Gamma-Glutamyl Transferase
HCC	Hepatocellular Carcinoma
HCV	Hepatitis C Virus
HCVpp	HCV Pseudotype Particle
HCV RNA	Hepatitis C Ribonucleic Acid

HFLC	Human Foetal Liver Cells
HIV	Human Immunodeficiency Virus
HKG	House Keeping Gene
HLA	Human Leukocyte Antigen
HVR 1	Hypervariable Region 1
HVR2	Hypervariable Region 2
IFN	Interferon
IFNAR1	Interferon Associated Receptor 1
IFNAR2	Interferon Associated Receptor 2
IL-2	Interleukin-2
IL-4	Interleukin-4
IL-10	Interleukin-10
IL-28B	Interleukin-28B
IRES	Internal Ribosomal Entry Site
IRF	Interferon Regulatory Factor
ISDR	Interferon Sensitivity Determining Region
ISG	Interferon Stimulated Gene

IVDU	Intravenous Drug Users
JAK	Janus Kinase
JFH-1	Japanese Fulminant Hepatitis-1
kb	Kilo bases
LDL	Low Density Lipoprotein
LDLr	Low Density Lipoprotein Receptor
LT	Liver Transplant
MC	Mixed Cryoglobulinaemia
MHC	Major Histocompatibility
MxA	Myxovirus Resistance Protein A
NA	Not Available
NANBH	Non-A non-B Hepatitis
NF κ β	Nuclear factor Kappa β
NHL	Non Hodgkin's Lymphoma
NK cells	Natural Killer cells
NHS	National Health Service
NICE	National Institute for Clinical Excellence

NS	Non Structural
NS2	Non Structural 2
NS3	Non Structural 3
NS4A	Non Structural 4A
NS4B	Non Structural 4B
NS5A	Non Structural 5A
NS5B	Non Structural 5B
nt	Nucleotide
NTC	No Template Control
NTR	Non Translated Region
ORF	Open Reading Framework
PAMP	Pathogen Associated Molecular Pattern
PBMC	Peripheral Blood Mononuclear cell
PBS	Phosphate Buffer Saline
PCR	Polymerase Chain Reaction
PE	Phycoerythrin
PEG-IFN	Polyethylene Glycol (Pegylated) Interferon

PHHs	Primary Human Hepatocytes
PIV5	Parainfluenza Virus 5
PIV5-V	Parainfluenza Virus 5 V protein
PKR	Protein Kinase R
qPCR	Quantitative Polymerase Chain Reaction
PRR	Pathogen Recognition Receptor
R	Receptor
RdRp	RNA Dependent RNA Polymerase
RIG-I	Retinoic Acid Inducible Gene-I
RNA	Ribonucleic Acid
RT	Reverse Transcriptase
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
RVR	Rapid Virological Response
SCID	Severe Combined Immune Deficiency
SOC	Standard of Care
SR-BI	Scavenger Receptor Class B Type 1

SSCP	Single Strand Conformational Polymorphism
ssRNA	Single Stranded Ribonucleic Acid
SVR	Sustain Virological response
TGF	Transforming growth factor
TLR	Toll Like Receptors
Treg	T Regulatory Cells
Tyk2	Tyrosine Kinase 2
VACV	Vaccinia Virus
WHO	World Health Organization

Chapter One

An Overview of Hepatitis C Virus

Infection and Replication

1.1. Introduction to hepatitis C infection

Infection with the hepatitis C virus (HCV) is emerging as a global health problem. It affects 130-185 million people worldwide and causes significant morbidity and mortality (Shepard, Finelli et al. 2005, Mohd Hanafiah, Groeger et al. 2013). It can lead to acute or chronic hepatitis, steatosis, cirrhosis, liver failure and hepatocellular carcinoma. In addition, HCV is implicated in extrahepatic sequelae like lymphoproliferative diseases and neurological defects (Ghinoi, Mascia et al. 2004, Monaco, Ferrari et al. 2012). Thus far there is no vaccine available to prevent HCV infection. Current therapy against hepatitis C¹ is non-specific, costly, prolonged, not suitable for everyone, poorly tolerated and often ineffective with only 50-60% patients achieving a sustained virological response (SVR) at the end of this regime (D'Souza and Foster 2004). Out of the 6 major genotypes of the HCV, genotype 1, 2 and 3 are most prevalent globally (Simmonds, Alberti et al. 1994). HCV genotype 1 is least responsive to interferon alfa and ribavirin therapy while genotype 3 has a higher relapse rate. Therefore, extensive research is underway to develop novel drugs against hepatitis C. Over the last few years two of these new agents, i.e.; telaprevir and boceprevir have been licensed and when combined with pegylated interferon and ribavirin they increase the proportion of people with genotype 1 HCV who respond to therapy. Other, more potent agents are at an advanced stage of development. The clinical introduction of new therapies for chronic HCV is hampered, at least in part, by the absence of a cell replication model for all

¹ At the time this work was initiated the standard of care for patients with hepatitis C was Pegylated interferon and ribavirin and here we describe conditions at the commencement of this work with updates of possible new agents listed where appropriate

viral strains of hepatitis C. The aim of this research was to develop novel replication models for HCV that will allow new drugs to be tested against all viral genotypes.

1.2. Overview of hepatitis C virus

1.2.1. Historical introduction

It is difficult to identify the origin of HCV since there are no stored blood samples older than 50 years to test for the presence of the virus. Since blood to blood contact is the main source of transmission, there is a debate as to whether the viral transmission has increased in the past hundred years during which time the virus has spread and evolved (Smith, Pathirana et al. 1997). However, it is unlikely that the origin of HCV will ever be formally proven and the historical transmission rates are likely to remain purely conjectural.

In the post world war II years it was noted that the proportion of patients receiving a blood transfusion developed hepatitis that appeared to persist. The infection was not caused by hepatitis A or hepatitis B and was therefore named non-A non-B hepatitis (NANBH). A putative cause for NANBH was identified in 1989 by antibody screening of a phage display library and was given the name hepatitis C virus (Choo, Kuo et al. 1989). Subsequent studies showed that this virus was responsible for most cases of NANBH.

Developments in diagnostics led to the evolution of effective tests and by 1992 blood tests that effectively eliminated the chances of the presence of

HCV from the blood transfusion supplies were developed (Fujiyama, Kawano et al. 1992). However, accurate Identification of HCV was challenging because of genetic heterogeneity, low titres in blood and the natural history of infection which involved a lengthy asymptomatic stage (Feitelson MA (2006) Hepatitis C Virus From Laboratory to Clinic. Cambridge University Press.). Sequence comparison with the RNA viruses showed that HCV encodes a conserved serine-like protease domain, a RNA polymerase domain and a helicase domain (Choo et al. 1991) and was classed as a Flavivirus.

1.2.2. Taxonomy, structure and biology

HCV is a positive-sense RNA virus classified in the *Flaviviridae* family and is the sole member of genus *Hepacivirus* (Simmonds, Alberti et al. 1994). It is detected in serum in cases of both acute and chronic infection and is closely related to yellow fever virus and dengue virus (Choo, Kuo et al. 1989). It is a hepatotropic, double-shelled, enveloped, virus, 50 to 60nm in diameter. It is believed to replicate mainly in the cytoplasm of hepatocytes while the replication in extrahepatic sites is hugely debated, (reviewed in Revie and Salahuddin 2011). It is transmitted from cell to cell and in the infected liver approximately 10^{10} - 10^{12} virus particles are produced which are not believed to be directly cytopathic (Choo, Kuo et al. 1989, Choo, Richman et al. 1991).

1.2.2.1. Non-translated regions

The hepatitis C viral genome is 9.6kb long. It contains highly structured and conserved 5' and 3' untranslated regions (5' and 3' UTRs) which regulate

genomic duplication and initiate translation and flank a larger open reading frame (ORF). The 5' UTR is relatively conserved and is approximately 341 nucleotides (nt) in length. It consists of four major structural domains. Domain I though not a part of IRES (internal ribosomal entry site) is important for replication (Tsukiyama-Kohara, Iizuka et al. 1992). Domain II and III are the secondary structures formed by the HCV IRES. They contain elements which are necessary for initiation of translation (Brown, Zhang et al. 1992). Domain IV contains the initiation codon (Hellen and Sarnow 2001). 3' un-translated region varies in length from 200 to 235nt and has a variable region, a poly U/UC stretch and a 98nt long conserved region (Tanaka, Kato et al. 1995, reviewed in Shi and Lai 2006).

1.2.2.2. Viral proteins (structural and non-structural)

The ORF encodes a 3000-3030 amino acids long polyprotein which is cleaved co- and post-translationally into 10 distinct structural and non-structural viral proteins. **Structural proteins** constitute the building blocks of the virus and are cleaved by the host proteases. They comprise **core** (C) and **envelope proteins** which are present at the amino-terminal. E1 (has 6 glycosylation sites) and E2 (11 glycosylation sites) are glycoproteins which are anchored in a lipid bilayer surrounding the nucleocapsid formed by the core protein and genomic RNA. Both core and envelope proteins are required for virion formation (Vassilaki, Friebe et al. 2008). E2 engages CD81 on host cell surface during viral entry process (Falkowska, Kajumo et al. 2007). Wakita et al. incubated the cell culture adapted viral strain JFH-1 with Huh7.5 cells with and without CD81 antibodies and observed that after 48

hours CD81 antibodies reduced the number of infected cells and HCV RNA within the cells, hence confirming the role of CD81 as a viral entry receptor (reviewed in Wakita, Pietschmann et al. 2005). However, other viral receptors have subsequently been identified and have a role in the entry process (discussed in section 1.8.1.2). At the N-terminus extremity, E2 contains a highly variable region of HCV genome called the hypervariable region (HVR-1) which is 27 amino acids in length (Weiner, Christopherson et al. 1991).

A 16 KDa protein called ARFP, (alternative open reading frame protein), F (for frame-shift) or +1 Core (indicating its position) formed by the frame-shift of core protein through an open reading frame overlapping core gene at +1 nucleotide, was identified for the first time in 1998 by Walewski et al. (Walewski, Keller et al. 2001). Antibodies against this protein have been identified and it is believed to be formed during the viral replication process but its exact role remains unclear. Furthermore, it is reported that this protein does not have a role in viral replication as halting its production does not have any negative effect on viral replication (Vassilaki and Mavromara 2009) and it is unclear whether this is an artefact or a viral protein of unknown function.

P7 is a 63-residue peptide that does not form part of HCV virion and is also cleaved by the host proteases (Reed and Rice 1998). It has ionic channel activity within lipid bilayer membranes (Carrasco 1995). In addition, p7 has a role in the assembly, release and production of infectious hepatitis C virions from liver cells (Steinmann, Penin et al. 2007, Jones, Murray et al. 2007). Mutations in the specific p7 sequence led to the loss of ion channel activity

(Murray, Jones et al. 2007). Moreover, certain drugs can inhibit its activity and show antiviral function (Pavlovic, Neville et al. 2003). The junctions between these proteins (C/E1, E1/E2, E2/p7 and p7/NS2) are cleaved by endoplasmic reticulum (ER) resident host signal peptidases (Wu 2001).

The **non-structural region** of hepatitis C virus comprises of proteins termed as **NS2, NS3, NS4A, NS4B, NS5A** and **NS5B**, which are cleaved from the polyprotein by the viral proteases (NS2/3 autoprotease and NS3/4A protease) and are functionally categorised as proteases (NS2, NS3 and NS4A), helicases (NS3) or RNA-dependent RNA polymerase (NS5B). This non-structural region plays a critical role in viral replication. NS2 and NS3 carry out auto-catalytic cleavage and thereby separate the NS2 protein from NS3. The NS2/NS3 junction cleavage is carried out between the carboxy NS2 terminal end and the amino NS3 end and is independent of the NS3 protease (Phan, Beran et al. 2009, Schregel, Jacobi et al. 2009).

NS3 has a diverse set of functions. It has protease activity in conjunction with NS4A, as well as RNA helicase and nucleotide triphosphatase (NTPase) activity. It thus plays a key role in HCV replication and morphogenesis (Kwong, Kim et al. 2000, Locatelli, Gosselin et al. 2001). After being cleaved from NS2, NS3 in conjunction with NS4A (NS3/4A serine protease) mediates successive proteolysis of NS3/NS4A, NS4A/NS4B, NS4B/NS5A and NS5A/NS5B junctions and play an active role in HCV non-structural protein maturation (De Francesco and Steinkuhler 2000). NS3 is also proposed to interfere in some cellular signal pathways and over rides normal cellular

defence functions making the cell more vulnerable to infection (Jouan, Melancon et al. 2010).

The main function of NS4 is to act as a co-factor for NS3 mediated proteolysis of non-structural viral polypeptide (reviewed in Tu, Ziemann et al. 2010). It also anchors NS3 to endoplasmic reticulum (ER) and plays a pivotal role in formation of the membrane associated replication complex (Lundin, Monne et al. 2003).

NS5 is processed into two proteins NS5A and NS5B. NS5A is a hyperphosphorylated protein and its role in determining sensitivity to IFN has been discussed through a region called interferon sensitivity determining region (reviewed in Gale, Korth et al. 1998, Lauer and Walker 2001). However, in a study of HCV-1b NS5A quasispecies no NS5A related sequence showed resistance or sensitivity to IFN (Pawlotsky, Germanidis et al. 1998). NS5A has been implicated in replication and is thought to act as an anchoring protein, locking the replication complex onto the endoplasmic reticulum (Enomoto, Sakuma et al. 1995). NS5B is a RNA-dependent RNA-polymerase (RdRp) which does not have 3-5 exonuclease proofreading activity. This lack of proof reading activity renders HCV replication error prone and subject to mutations and hence evolution of different viral subtypes (Lohmann, Roos et al. 2000). The virions of HCV associate with serum low density and very low density lipoproteins. This association can create a wide range of viral particles with variable buoyant densities. Viruses with lowest density are most infective (Yamamoto, Aizaki et al. 2011). However, this variability has made it difficult to obtain precise structural

information about this virus (reviewed in Rice 2011). Host cells which permit HCVpp (pseudotype particles that display functional E1 E2 glycoprotein complexes) entry and are of hepatic origin express low density lipoprotein receptor (LDLr), CD81 and scavenger receptor class B type I (SR-BI). Though other cell types also express these receptors but are either not permissive or poorly permissive to HCVpp entry and infection.

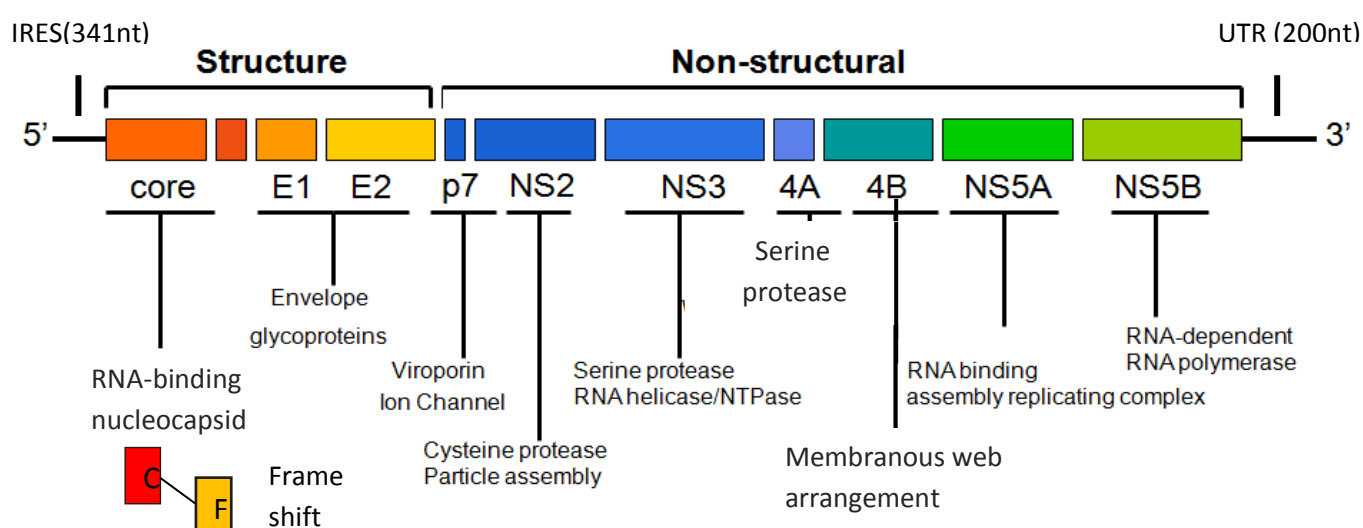


Figure 1.1: Open reading frame (of HCV genome) encoded proteins and their functions: Consisting of two regions, 1. Structural (comprising of core (C) and envelop proteins E1 and E2) and 2. Non-structural region (composed of NS2, NS3, NS4A, NS4B, NS5A and NS5B).

1.2.3. Life cycle of hepatitis C virus

HCV interacts with a number of host factors for completion of its life cycle. The complete HCV life cycle was studied in detail after the advent of JFH1, a unique viral strain that infects and completes a full replication cycle in cultured hepatocytes (Lindenbach, Evans et al. 2005). During its replication

cycle HCV undergoes various stages which encompass viral attachment, ingress into host cells and fusion, viral RNA translation, post-translational processing, replication, and viral assembly and release.

The early stages of viral life cycle involve attachment of HCV to the host cell surface receptors and ingress into the host cell through receptor mediated endocytosis. The viral and host factors which interact together to carry out this process are envelope glycoproteins E1 and E2 of HCV and host cellular molecules including glycosaminoglycans, tetraspanin CD81, scavenger receptor B type 1, claudin-1, dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin (DC-SIGN or CD209), liver/lymph node-specific intercellular adhesion molecule-3 (ICAM-3)-grabbing integrin (L-SIGN or CD209L), low density lipoprotein receptor and asialoglycoprotein receptor (reviewed in Pawlotsky, Chevaliez et al. 2007). The precise details of how receptor engagement leads to internalisation and release of viral nucleic acid remain elusive. It is suggested that HCV enters a cell by clathrin-mediated endocytosis after which viral envelop uncoating and release of the single stranded HCV genome (messenger sense viral RNA) into host cell cytoplasm takes place. This is followed by translation of HCV proteins via the IRES locus at the 5'UTR region. Viral proteins and genomic RNA template form viral replication complexes which assemble in the membranous web formed by NS5B protein on endoplasmic reticulum (ER). NS5B protein then acts as an enzyme (RdRp) which produces anti-genomic strand which subsequently produces new genomic strands. Genomic strands either translate more viral proteins, form anti-genomic RNA or get packaged into new viral particles which egress out of the cell through the secretory pathway

(reviewed in Weber, Wagner et al. 2006). Egger et al. observed that HCV proteins induce distinct alterations to the ER membranes producing a membranous web which is vital for HCV proliferation (Egger, Wolk et al. 2002). During HCV RNA replication double-stranded (ds) RNA intermediates are produced. The dsRNA acts as pathogen associated molecular patterns (PAMPs) which binds with many innate immune receptors called pathogen recognition receptors (PRRs) e.g.; RIG-1 and mda-5 (discussed later) (reviewed in Li and Lemon 2013).

The virus genetic material is translated by host ribosomes encoding a long open reading frame and polyprotein formation which is then processed by host and viral proteases into the ten individual gene products (discussed earlier) and subsequent production of a replication complex. HCV genomic replication takes place through a full-length negative-stranded intermediate which serves as a template for *de novo* production of positive stranded RNA. The synthesis of these RNAs takes place by viral RdRp (NS5B) (reviewed in Penin, Dubuisson et al. 2004, Moradpour, Penin et al. 2007, Bartenschlager and Lohmann 2000). As discussed previously because of the lack of NS5B proofreading ability, there is a high rate of mutation in the HCV genome leading to production of quasispecies.

The final stage of HCV life cycle is not well understood and it is thought that the structural part of HCV-core protein (C) and two envelope glycoproteins (E1 and E2) along with the non-structural components- P7, NS2, NS3 and NS5B are packaged together and contribute to the formation of infectious virions. These infectious virions are thought to egress through the cell after

budding into the ER lumen and are expelled through the host cell secretory system (reviewed in Pawlotsky, Chevaliez et al. 2007). Assembly and release of HCV is associated with the lipoprotein pathway (reviewed in Zeisel, Fofana et al. 2011).

Several host factors are required for the infection and replication of HCV in the hepatocytes. Cyclophilins are family of proteins which have peptidyl prolyl isomerase activity leading to protein folding by isomerization of peptide bonds from *trans* to *cis* form at the proline residues. Cyclophilin A is a cytosolic protein implicated in replication of HCV (Kaul, Stauffer et al. 2009). It has been observed that cyclosporine; an immunosuppressant agent which is known to bind with cyclophilin inhibits HCV replication. In one study it was observed that cyclosporine inhibited incorporation of NS5B into the HCV replication complex and hence inhibited HCV replication by preventing formation of the replication complex (Liu, Yang et al. 2009). Moreover, variants of cyclophilin A did not allow infection and replication of HCV (von Hahn, Schiene-Fischer et al. 2012).

MiR-122 is a microRNA which is known to assist in HCV replication. The exact mechanism as to how it exerts its pro-replication function is not fully elucidated. It binds with the 5' UTR region of the viral genome at two sites and promotes HCV RNA replication (Jopling, Yi et al. 2005). In one study, Shan et al. demonstrated that miR-122 triggers HCV replication by post-transcriptional suppression of haeme oxygenase enzyme synthesis (Shan, Zheng et al. 2007). Furthermore, it was shown by Li et al. that miR-122 protects HCV genome against degradation by the RNA exonuclease Xrn-1, in

the cytoplasm of host cells (Li, Masaki et al. 2013). However, it is suggested that this may not be the only way by which this microRNA promotes the HCV life cycle. Hence, miR-122 may have yet another function in HCV replication (reviewed in Garcia-Sastre and Evans 2013).

1.3. Epidemiology and global burden of hepatitis C

1.3.1. Prevalence and incidence estimates

A large global population is already infected with HCV posing a major setback in controlling this infection. As the majority of those infected belong to the developing countries the global burden of disease and relative cost estimates are difficult. The latest figures from the WHO, revised in 2005, showed that 185 million people are positive for HCV antibody globally or 2.8% of the world's population is infected, in contrast to 1990 when estimates showed 122 million or 2.3% individuals being affected worldwide. 130-170 million are thought to have chronic infection (Te and Jensen 2010, Mohd Hanafiah, Groeger et al. 2013). Most data on global HCV prevalence derives from hepatitis C seroprevalence studies which are generally cross-sectional in design and involve only selected groups e.g. patients with liver disease or blood donors which may not represent the whole community. As compared to developed countries, developing nations have a wide variation in their prevalence estimates and fewer data to support assumptions about disease burden in these regions making data on the prevalence in these countries much less accurate (Shepard, Finelli et al. 2005). Incidence rates of hepatitis C can give an estimation of the future burden of disease, however it is

difficult to distinguish the acute form of HCV infection from chronic or resolved infection (reviewed in Shepard, Finelli et al. 2005, Lavanchy 2009). Egypt has highest national prevalence rates and of those born before 1960, 50% carry HCV, which was spread after a campaign to eradicate schistosomiasis intravenously to millions between 1950 - 1980 (Frank, Mohamed et al. 2000, Strickland 2006).

In the United States it was estimated that 3.9 million people were infected with HCV according to reports from National Health and Nutrition Examination Survey (NHANES) from blood collected during 1988-1994 from persons representing households (not including homeless people and prisoners) (Armstrong, Wasley et al. 2006). Ethnic minorities were shown to harbour HCV more often than the indigenous population. The HCV report by NHANES in 2000 suggests that in non-Hispanic blacks HCV prevalence was greater than in the 'white' population by 14%. The main risk factor for contracting HCV in developed nations is injecting drug use (IDU) (Williams, Bell et al. 2011) and most of the epidemiological estimates come from the developed world where chronic HCV is considered as a disease of drug users. Nonetheless, worldwide other risk factors clearly play a role in HCV transmission (see later) although injection drug use continues to play a major role even in the developing world - it has been estimated that out of 13.2 million IVDUs, 10 million reside in developing nations and only 22% belong to developed nations (reviewed in Chu, Chiang et al. 2009).

In the UK, general population studies have not been performed and most prevalence studies focus on certain groups such as IDUs. In England and

Wales in a study testing 1034 drug users for HCV by using dried blood spot for diagnosis, it was found that 32% had the disease (Hickman, McDonald et al. 2008), while in another study looking at 1949 IDUs in Glasgow, salivary antibodies against HCV were positive in 61% (Taylor, Goldberg et al. 2000). In a study looking at 4729 pregnant women at a London hospital between 1997-1999, showed prevalence in that group of 0.8%. This was a relatively small scale study and chances of selection bias with women self presenting to the clinic was high. Moreover, pregnant women represent a low risk group and might not represent the whole population (Ward, Tudor-Williams et al. 2000). In a study looking at prevalence of HCV in immigrants from South Asian countries, it was found that Pakistani born individuals had a prevalence of 2.7% while in Bangladeshis prevalence was 0.44% (Uddin, Shueb et al. 2010). In 2010, attempts were made to collate all the available data for England and Wales and showed that an estimated 191,000 (95% CI: 124000-311000) people carried antibody against HCV, out of which 142,000 (95% CI: 90000-231000) had chronic infection. However, the confidence intervals were broad and overall prevalence still remains unclear due to unavailability of data related to ethnic minorities (reviewed in De Angelis, Sweeting et al. 2009).

In France, a screening study carried out within general practices involving 11805 individuals showed a prevalence rate of 1.3% (Pradat, Caillat-Vallet et al. 2001). A study done in Southern Italy on 1352 persons showed a higher rate of 12.6% (Guadagnino, Stroppolini et al. 1997). In a selected population study in Germany with 2796 dialysis patients, a prevalence of 7% was seen (Hinrichsen, Leimenstoll et al. 2002) while in a multi-centric study involving

984 dialysis patients in France, 23.6% had HCV infection (Dussol, Berthezene et al. 1995). In Egypt overall prevalence rates vary from 15-20% (Frank, Mohamed et al. 2000). The prevalence in Pakistan is believed to be high - in the total population of 170 million in Pakistan it is estimated that 10 million (6%) people harbour HCV infection (Waheed, Shafi et al. 2009). Nonetheless, a cross sectional study in healthy children showed a prevalence of 0.44% indicating that there could be variation of HCV infection among various age groups (Agboatwalla, Isomura et al. 1994).

In summary, hepatitis C infection poses a considerable global health problem because it establishes persistent infection in its human host and infection cannot be prevented as a vaccine is not yet available. Neal et al. have demonstrated that HCV infected individuals have a three times higher death rate compared to uninfected people (Neal, Trent Hepatitis et al. 2007). Chronic HCV infection is currently believed to be responsible for considerable mortality worldwide with an estimated 1.4 million deaths per year, out of which 796,000 are due to cirrhosis and 616,000 are due to primary liver cancer (reviewed in Seeff 2002).-

1.3.2. Genotypes and distribution

Hepatitis C is a highly variable virus but it is now recognised that the viral variants can be classified into different genotypes. Six major genotypes of HCV were identified through phylogenetic analysis of genes encoding NS5 proteins of HCV and comparison with the published sequence led to the identification of different types and subtypes of HCV which vary from each other by 33% over the whole genome (Simmonds, Holmes et al. 1993).

Knowing the epidemiology of genotypes is important in order to track HCV infection back to its source and to predict response to therapy. Genotype 1, 2 and 3 are broadly distributed globally. Genotypes 1a and 1b account for 60% of infections worldwide with genotype 1a most prevalent in north of Europe and America while genotype 1b predominates in south and east of Europe and in Japan. Genotype 2 is mostly found in Europe, Mediterranean countries and Far East and genotype 3 is common in South-East Asia. The Middle-East, Egypt and Central Africa have been affected mostly by genotype 4 while genotype 5 has been reported in South Africa. Other genotypes are distributed mostly in Asian countries (reviewed in Hnatyszyn 2005, Simmonds, Bukh et al. 2005) .

Some studies report that there might be a relationship between the type of genotype affecting the host and the progression of disease (Gane, Portmann et al. 1996, Kao, Chen et al. 1995, Gonzalez-Michaca, Mercado et al. 2000). It has been suggested that genotype 1b leads to a more aggressive disease and is also associated with more severe graft injury in patients who undergo transplantation (Gane, Portmann et al. 1996). Moreover, HCV genotyping is of clinical significance as response to treatment varies among different strains and decisions about length of treatment is tailored according to the viral genotype (reviewed in Narahari, Juwle et al. 2009, Yu and Chuang 2009). It is well established that patients infected with genotype 1 show the poorest response to conventional antiviral treatment with pegylated interferon (PEG-IFN) and ribavirin compared to infection with other genotypes (Cheng, Roberts et al. 2010).

1.4. Disease transmission patterns

HCV is a blood borne hepatotropic pathogen which is transmitted through blood to blood contact. In developed countries till 1980 the main source of HCV transmission was exposure to contaminated blood or blood products and injecting drug use. Following the introduction of routine screening of donor blood, blood products and organ transplants, illicit use of injectable drugs remains the main source of HCV transmission in the developed world (Donahue, Munoz et al. 1992). The acquisition of HCV infection is more frequent compared to HBV and HIV infection in injecting drug users (reviewed in Wasley and Alter 2000), perhaps because of effective vaccination programs for HBV and because HIV is much less infectious through this route compared to HCV (reviewed in Beltrami, Williams et al. 2000).

In developing nations unsafe use of therapeutic procedures and transfusion of unscreened blood or blood products is still a significant cause of HCV transmission. Needle reuse for therapeutic or preventive procedures is common in the Middle-East, South-East Asia and Western Pacific (Hutin and Chen 1999, Kane, Lloyd et al. 1999). Due to a lack of a nationwide system for blood screening in developing countries a significant number of individuals continue to be infected with HCV every year (Simonsen, Kane et al. 1999).

Occupational exposure to HCV is seen in health-care workers who have sustained a contaminated needle stick injury and the observed attack rates in these cases are as low as 0.3%, although a few studies have estimated it to be higher, at around 3-7% (Martins, Almeida et al. 1996). HCV is less likely to

be transmitted through mucosal contact, making sexual transmission and vertical transmission relatively uncommon. However, in some cases transmissions have reported from patients who are co-infected with HIV (Matthews, Hellard et al. 2007, Polis, Shah et al. 2007) and this seems particularly common in men who have sex with men. Materno-foetal HCV transmission occurs in some 4-10% of cases and is common when the mother is co-infected with HIV, particularly in those delivered vaginally. (Paccagnini, Principi et al. 1995, (Zanetti, Tanzi et al. 1995, Polywka, Feucht et al. 1997, Granovsky, Minkoff et al. 1998).

1.5. Natural history of hepatitis C infection

1.5.1. Acute infection

Hepatitis C infection has vast heterogeneity in its clinical course, progression and outcome (reviewed in Alberti, Vario et al. 2005). Early infection with HCV is mostly asymptomatic leading to an inability to precisely identify the time of acquisition of disease, but in a few cases severe acute infection takes place with a rise in serum transaminases and the appearance of jaundice. Occasionally acute hepatitis C may resolve spontaneously without long term sequelae. Epidemiological studies show that an estimated 15-20% of all infected persons resolve their infection without undergoing any treatment (reviewed in Katze, He et al. 2002, Sumpter, Loo et al. 2005). However, there are reports that suggest that resolution of the acute stage of HCV infection could range from 10 to 50% and that several cofactors predict the severity of liver disease secondary to HCV. Those exposed to low levels of HCV virus

may resolve without clinical signs of any disease and it is suggested that T cell immunity might develop even without anti-HCV seroconversion in such cases (reviewed in Alberti, Chemello et al. 1999). In a study looking at 60 patients with acute HCV infection, 44% spontaneously cleared the virus (Gerlach, Diepolder et al. 2003). In a prospective study 24% of the 41 patients followed for 6 years cleared the virus spontaneously (Barrera, Bruguera et al. 1995). Various host factors like increased age at the time of infection, male gender, co-infection with HIV or immunosuppressed individuals, IV drug use, ethnic background (such as African Americans), co-morbidities (i.e. Haemochromatosis, NASH) contribute to progression to chronic disease (Thomas, Astemborski et al. 2000). However, all of the studies to-date examining the transition from acute to chronic infection have been relatively small and the precise factors that determine the outcome of acute infection remain unclear.

About 20-30% of acutely infected individuals may become symptomatic within 3-12 weeks of infection (reviewed in Romero-Gomez, Del Mar Vilorio et al. 2005). HCV RNA starts rising within 1-2 weeks of exposure to HCV with a subsequent rise in serum transaminase levels sometimes up to 10 times the upper limit of normal along with the appearance of clinical symptoms like malaise, anorexia, jaundice and weakness. Acute infection can very rarely progress to fulminant liver disease (reviewed in Romero-Gomez, Del Mar Vilorio et al. 2005).

In self-limited acute hepatitis C infection, symptoms can last for a few weeks after which there is a drop in serum transaminases and HCV RNA levels and

anti-HCV levels appear with clearance of infection. It is thought that the presence of jaundice increases the chance of spontaneous viral clearance (reviewed in Tillmann and Manns 1996, Lehmann, Meyer et al. 2004).

1.5.2. Chronic infection

Chronic HCV infection is characterised by the inability to clear HCV leading to raised aminotransferases or persistent viremia for more than 6 months. About 80% of exposed individuals are believed to develop a chronic infection (reviewed in Conry-Cantilena, VanRaden et al. 1996, Alter, Kruszon-Moran et al. 1999). Liver function tests and serum transaminases fluctuate considerably during the course of chronic hepatitis C thus they cannot be considered as reliable markers of disease progression. Liver biopsy is currently the gold standard for the staging and grading of chronic hepatitis C infection.

In the patients persistently infected with hepatitis C initially the symptoms are usually mild and it takes decades before the serious consequences of chronic HCV infection become apparent. The long term complications of chronic HCV infection (either liver cancer or decompensation) may lead to indications for liver transplantation (Brown 2005).

In the patients who develop chronic hepatitis C there is 15-20% progression to cirrhosis over 20 years (Alberti, Chemello et al. 1999, Hissar, Kumar et al. 2009). The proportions of patients who will develop cirrhosis after longer periods of infection are unknown but probably substantially greater than 20%. One study, in a self-selected group of patients, suggested that up to 70% of

patients may develop cirrhosis after 70 years of persistent infection (D'Souza, Glynn et al. 2005). In a study done in Austria, 30 patients who contracted HCV in a plasmapheresis unit were followed for 18 years, variable rates of fibrosis progression were observed in different age groups and an average of 20% patients progressed to cirrhosis. It was seen that increased age at the time of acquisition of HCV infection was an important host factor causing progression to cirrhosis (Datz, Cramp et al. 1999). A systematic review of 111 studies including a total of 33121 individuals with chronic HCV infection has reported that 16% of the patients developed cirrhosis after 20 years of chronic infection (Thein, Yi et al. 2008).

In the patients with cirrhosis every year approximately 4% develop hepatocellular carcinoma (HCC) and 1-3% develop decompensated liver disease while death occurs in 1.3-3.7% per year (reviewed in Hissar, Kumar et al. 2009). HCC is very common globally (it is the third most common cause of cancer related deaths worldwide) and currently it is estimated that chronic HCV infection is a major cause of this cancer with 40.1% of patients in one study being anti-HCV positive (Levrero 2006). For this reason it is recommended that patients with established cirrhosis should have 6 monthly ultrasonography and alpha-fetoprotein monitoring.

Before the discovery of hepatitis C virus in 1980s there was a high incidence of infection through blood transfusions and injecting drug use and as chronic hepatitis C related morbidity takes decades to develop, it is highly expected that the burden of disease will dramatically rise in coming years (reviewed in Grebely and Dore 2011)

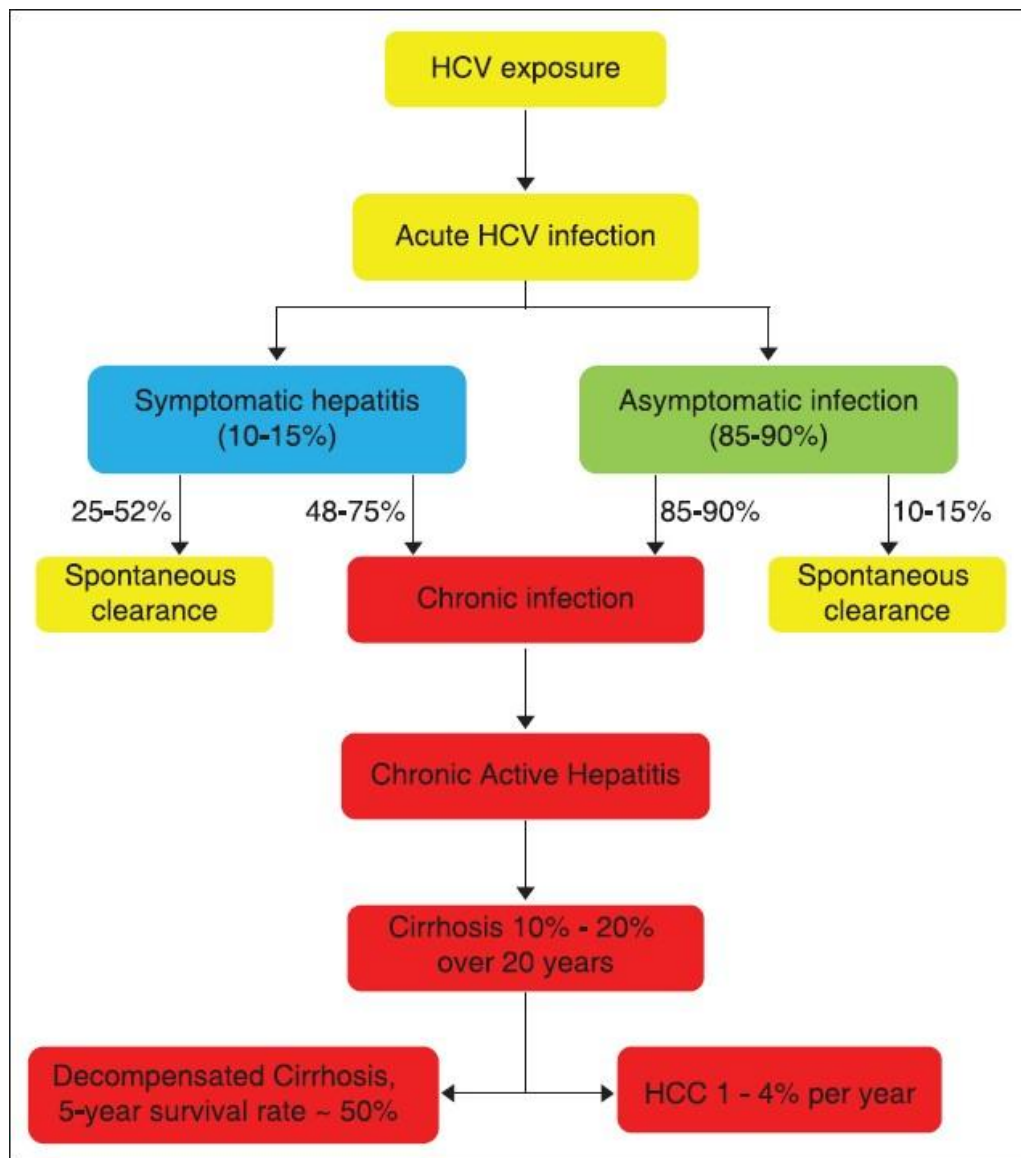


Figure 1.2: Natural history following infection with hepatitis C virus (Gupta, Bajpai et al. 2014)

In the chronic HCV infected patients the course of disease is extremely variable and fibrosis progression can only be partially predicted by different cofactors like the age at infection, disease duration, ALT profile and stage of fibrosis and liver histology.

1.5.3. Single nucleotide polymorphisms on the IL28B locus

In 2009, several genome wide association (GWAS) studies (used to identify genetic variants linked with complex traits) recognised two single nucleotide polymorphisms on the IL28B gene locus. These common variants are rs12979860 CC and rs8099917 TT (Thomas, Thio et al. 2009). It was found that these variants were linked with clearance of HCV, response to therapy and progression of disease course (Rauch, Kutalik et al. 2010). It was observed that patients with CC variant responded well to the standard of care (SOC) therapy which was PEG-IFN and ribavirin at that time. In contrast CT or TT variants responded less efficiently (Ge, Fellay et al. 2009, Tanaka, Nishida et al. 2009).

1.5.4. Clinical features and extrahepatic manifestations

HCV's major weapon is its silence, most patients remain asymptomatic for considerably long periods of time and even in the acute infection, jaundice appears in only 25-30%. Most symptoms such as low grade fever, anorexia, abdominal pain, fatigue, malaise, arthralgias and myalgias are non-specific. Acute symptomatic illness appearing in 20-30% can last for about 3-12 weeks and displays elevated serum aminotransferases (reviewed in Alter and Seeff 2000, Thimme, Oldach et al. 2001, Chen and Morgan 2006). Symptoms of liver failure and decompensation only develop in chronic HCV infection after many years of apparently 'asymptomatic' infection and these symptoms involve the development of ascites, upper gastrointestinal

bleeding secondary to varices or gastropathy, hepatorenal syndrome and hepatic encephalopathy.

Extrahepatic manifestations of hepatitis C are have been debated extensively. They can be the first sign of hepatitis C infection due to silent nature of the disease. EHMs can be seen in those with normal transaminases. Some studies suggest that between 40-74% of patients with hepatitis C infection develop at least one extrahepatic manifestation (EHM) during the disease course (Cacoub, Poynard et al. 1999, Cacoub, Renou et al. 2000). A decision about HCV therapy may be based on the presence of EHM (Galossi, Guarisco et al. 2007).

An association between HCV and EHM was first illustrated by Pascual et al. in 1990 when he described two patients with mixed cryoglobulinemia (MC). MC is the most studied EHM of HCV infection. It is a systemic vasculitis involving kidneys, skin and nervous system (Pascual, Perrin et al. 1990). EHM could be secondary to the formation of immune complexes and autoimmune phenomenon (Agnello and De Rosa 2004). Moreover, lymphotropism of HCV is widely accepted and could explain the pathogenesis of some haematological EHMs (Ferri, Monti et al. 1993).

Other common EHMs are Non-Hodgkin lymphoma, porphyria cutanea tarda, lichen planus, thyroid disease (usually hypothyroidism), rheumatology symptoms (arthritis, fibromyalgia, vasculitis and 'pseudo-Sjogren syndrome') etc (Galossi, Guarisco et al. 2007). It has also been observed that diabetes mellitus is more common in HCV patients compared to general population (Mason, Lau et al. 1999, Galossi, Guarisco et al. 2007) and is found to be

secondary to insulin resistance (Petit, Bour et al. 2001). Mason et al. suggested that HCV is a risk factor for DM which is unrelated to the severity of liver disease (Mason, Lau et al. 1999). However, some suggest that DM in HCV increases the risk of advanced fibrosis of the liver (Caronia, Taylor et al. 1999).

1.6. Hepatitis C management

Liver biopsy is the gold standard test to look at the extent of hepatic fibrosis and grading and staging of liver disease in chronic HCV patients. Some alternative tests are fibro scan, fibro test and enhanced liver fibrosis (ELF) test. Standard of care (SOC) antiviral therapy for HCV at the time that this work was completed showed success in 50-80% of patients with clearance of HCV RNA from serum, often accompanied by normalizing of liver biochemical tests and improvement of liver histology (Lindsay 2002). A patient is considered to have achieved a sustained virological response (SVR) when the serum remains negative for HCV RNA for a period of 6 months post-treatment (Lindsay 2002). The recurrence rate after gaining SVR is reported as 2% at 1 to 4 years after treatment ((Fujii, Itoh et al. 2010, Morgan, Ghany et al. 2010), but some of these 'late relapses' may represent new infections and most physicians regard an SVR as equivalent to a cure. Previous SOC antiviral treatment consists of combination therapy with pegylated Interferon alfa (2a or 2b) which is given once a week plus ribavirin which is taken daily (800mg for HCV genotypes 2 and 3 infected patients and 1000/1200mg for HCV genotype 1, 4, 5 and 6) (Hadziyannis, Sette et al. 2004, Fried, Shiffman et al. 2002). Response to therapy depends on both

viral- and host- associated factors. Various genotypes of HCV showed differences in response to this therapy when compared with each other thus genotypes are the most important predictors of response to therapy and determine the length of treatment duration and the dose of ribavirin. Genotype 2 and 3 are more responsive to therapy with almost 80% achieving an SVR while genotypes 1 achieve an SVR in no more than 40-50% of the treated patients (reviewed in Munir, Saleem et al. 2010, Cheng, Roberts et al. 2010). However, for patients with advanced disease these response rates may be considerably reduced – for example in patients with genotype 3 HCV who have cirrhosis, success rates with therapy may be less than 60% (Freshwater, O'Donnell et al. 2008, Sood, Midha et al. 2008, David, Rajasekar et al. 2010). The host factors which predict response to treatment include female gender, young age, low body mass index, low fibrosis score on biopsy and lack of co-morbidities such as drug and alcohol use, renal disease, co-infection with HIV or HBV (reviewed in Feld and Hoofnagle 2005).

The primary aim of therapy is viral eradication and important secondary goals are increased longevity, reduced liver damage and fibrosis progression, normalized biochemical liver tests, decreased incidence of hepatocellular carcinoma and lower mortality and morbidity. Previous SOC therapy recommends continuing treatment for 24 weeks in HCV genotype 2 and 3 patients and for 48 weeks in cases of HCV genotype 1, 4, 5 and 6 patients. It is recommended that treatment should be terminated if patients infected with HCV genotypes 1, 4, 5, and 6 do not show a two \log_{10} drop in their HCV viral load by the twelfth week of treatment as it is highly unlikely that they will

achieve SVR if therapy is continued (Lukasiewicz, Gorfine et al. 2010). However, there is no recommendation for treatment discontinuation secondary to lack of such a drop in viral loads in patients with genotypes 2 and 3 (Zeuzem, Rizzetto et al. 2009). For patients with HCV genotype 1 infection responding very rapidly to therapy- rapid responders (defined as undetectable virus after 4 weeks of therapy) there are various studies which indicate that 24 weeks therapy may be sufficient to achieve an SVR, (Jensen, Morgan et al. 2006, reviewed in Poordad, Reddy et al. 2008). However, these are relatively small scale studies and no properly powered assessment of short duration therapy has been reported to-date.

Interferon exerts its antiviral function through induction of interferon stimulated genes (ISGs) and establishes antiviral state in the cells. Interferons also stimulates T cell and NK cell activation, T cell proliferation and maturation of dendritic cells (reviewed in Feld and Hoofnagle 2005). The exact mechanism of action of ribavirin is still not clear, it is reported that ribavirin interacts with viral replication and increases the error frequency of NS5B mediated RNA transcription and hence leads to production of non-viable viral genomes (Crotty, Maag et al. 2000). It is still not clear whether the treatment of HCV infection leads to successful elimination of HCV, or whether there is persistence of small quantities of viral RNA (Radkowski, Gallegos-Orozco et al. 2005) in some sanctuary sites - so called 'occult' HCV.

There have been two recent advances over the last year in the treatment of chronic HCV infection and these have changed the optimal treatment of

genotype 1 HCV infection. Inhibitors of the NS3/4A serine protease i.e.; boceprevir and telaprevir have shown to be potent inhibitors of HCV genotype 1 replication and have improved SVR rates considerably both in patients who were not treated previously and those who previously failed to respond to pegylated interferon and ribavirin (Bacon, Gordon et al. 2011, Jacobson, McHutchison et al. 2011, Poordad, McCone et al. 2011, Zeuzem, Andreone et al. 2011).

The recent treatment recommendations entail the use of boceprevir or telaprevir in combination with pegylated interferon alfa and ribavirin (triple therapy) and these drugs should not be used in isolation and are only effective and licensed for patients with genotype 1 HCV (Ghany, Nelson et al. 2011). For treatment naïve patients, the dose of boceprevir is 800 mg three times a day together with pegylated interferon alfa and weight-based ribavirin (weight-based dose of ribavirin starts at 800 mg/day for patients weighing under 65 kg, and increased by 200 mg/day for each additional 20 kg of weight up to a maximum dose of 1400 mg (Nunez, Miralles et al. 2007)) for 24-44 weeks and this regimen is preceded by 4 weeks of lead-in treatment with PEG-IFN alfa and ribavirin alone (Kwo, Lawitz et al. 2010). In non cirrhotic patients shorter duration of treatment (28 weeks) can be considered if the HCV RNA level at the 8th and 24th week of therapy is undetectable while in cases where at the 12th week HCV RNA is >100 IU/ml treatment should be terminated as it is futile (reviewed in Poordad, McCone et al. 2011). The telaprevir treatment regimen consists of taking telaprevir 750 mg thrice daily with food (not low-fat) along with PEG-IFN alfa and weight-based ribavirin for 12 weeks followed by an additional 12-36 weeks of PEG-IFN alfa and

ribavirin. An additional 12 weeks is used for non cirrhotic patients whose HCV RNA level at weeks 4 and 12 is undetectable. These patients are defined as showing an extended rapid virological response and require shorter treatment durations of a total of 24 weeks. All other patients should receive total treatment duration of 48 weeks. (Zeuzem, Andreone et al. 2011). However, cirrhotic patients should be treated with telaprevir in combination with PEG-IFN and ribavirin for 12 weeks followed by a further 36 weeks of PEG-IFN and ribavirin without telaprevir (Ghany, Nelson et al. 2011) even if they have evidence of an early virological response. Similarly, treatment should be stopped if the HCV RNA level is $>1,000$ IU/ml at 4th or 12th week of therapy and/or is detected at 24th week of treatment (Ghany, Nelson et al. 2011). In patients who have received treatment beforehand and have relapsed or responded partially to previous SOC therapy re-treatment with triple therapy (either telaprevir or boceprevir along with PEG-IFN alfa and ribavirin) is recommended (Bacon, Gordon et al. 2011, Zeuzem, Andreone et al. 2011). As for null responders this treatment might also be considered although response rates are low. Furthermore, response guided therapy should not be considered in non responders to a previous course of therapy (Bacon, Gordon et al. 2011, Zeuzem, Andreone et al. 2011).

Patients failing to have a virological response, those who experience virological breakthrough (>1 log increase in HCV RNA) or relapse on one protease inhibitor should not be re-treated with the other protease inhibitor because of the possibility of viral resistance (Sarrazin, Kieffer et al. 2007, Sarrazin and Zeuzem 2010). IL28B genotype should be considered as a pre-treatment predictor of SVR for both previous SOC and triple therapy in

patients infected with genotype 1 strain of HCV if the patient or provider wishes to have information on the probability of response to treatment or on the probable duration needed for the treatment as likelihood of viral clearance with antiviral therapy or spontaneous resolution of HCV viral infection depends upon the nucleotide sequence near the gene for IL28B (discussed above in section 1.5.3) (Ge, Fellay et al. 2009, Thomas, Thio et al. 2009). However, it was shown that not all patients with CC variant responded and those with CT or TT variants could also attain SVR. Moreover, 53% of patients with CC variant and 28% of patients with TT or CT variants respond to therapy according to one series (Thomas, Thio et al. 2009). There have been reports that in the new SOC therapy using triple regimen (using protease inhibitors along with PEG-IFN and ribavirin), response could be predicted with IL28B status. However, the link is not as strong as with the previous SOC therapy (Akuta, Suzuki et al. 2010). Thus, it is highly debatable whether IL28B genotyping should be used to avoid unnecessary treatment with new SOC triple therapy or treatment should be given only depending upon the favourable IL28B genotype ignoring other variables like age, gender, ethnicity, fibrosis, viral genotype or compliance.

1.7. Host immune responses to HCV infection

Clearance of HCV infection relies on the T cell responses, innate immunity and on the direct antiviral effects of Interferon. The immune responses are orchestrated in a perfectly timed manner in order to clear the virus. Understanding of host immune responses to HCV infection and its evasion from these protective mechanisms demonstrate how the virus persists in its

host and may remain so even after treatment. Some of the strategies to escape the immune response by HCV are through retarding the interferon framework, suppression of antigen presentation, debilitating the T-cell response, development of peripheral tolerance, infection of immunologically privileged areas, down-regulation of HCV gene expression and viral mutations that abolish, anergise or antagonise antigen recognition by virus-specific T cells. In short, it is the rapid virus dynamics which surpass the immune strategies of the host (Missale, Cariani et al. 2004).

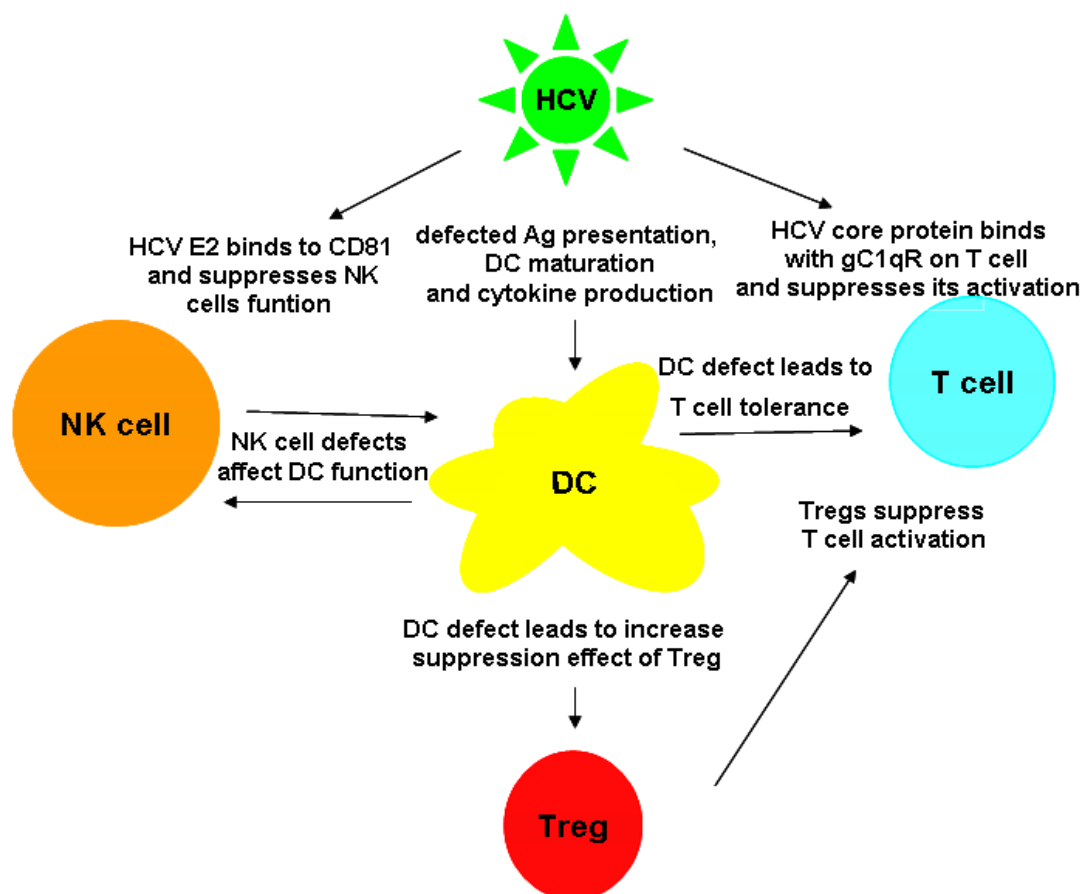


Figure 1.3: Mechanisms of evasion of immune effectors of the host by HCV.

1.7.1. Role of innate immune response to HCV infection

According to epidemiological estimates 20% of all infected persons resolve hepatitis C infection spontaneously without undergoing any treatment (reviewed in Sumpter, Loo et al. 2005). The precise mechanisms of viral clearance are not clear but probably involve a variety of different host defences. Innate immune response provides the primary line of defence against pathogens which are present in the host since birth and are antigen non-specific. Innate immune response 'buys time' for the host to set up adaptive immunity against the pathogen. Physical barriers like skin, mucous membranes, the complement system and cells such as granulocytes, macrophages, natural killer (NK) cells and antigen presenting cells like dendritic cells (DCs) and various molecules secreted by these cells, all constitute host innate immune responses (Beutler 2004). The major innate response to a viral infection is the production of Interferons (IFN) and inflammatory cytokines and chemokines. IFN acts by inducing ISGs and either acts directly in infected cells or indirectly by protecting uninfected cells. It is also pivotal for activation of variety of immune cells which help in viral clearance and surveillance (Borden, Sen et al. 2007). Moreover, inflammatory cytokines and chemokines attract T cells into the liver in HCV infection (Wald, Weiss et al. 2007, Zeremski, Petrovic et al. 2007). Evidence shows that HCV disrupts signal pathways involved in the production of IFN and cytokines (discussed in section 1.7.5).

IFN also has immunomodulatory function and up-regulates the expression of MHC class I and II molecules and in turn enhances cellular immune response

to viral infection *in vivo*. In some cases this might be seen later at the stage of recovery from viral infection rather than being an initial host defence mechanism. Other cells of the innate immune system like NK cells, natural killer T-cells and myeloid cells present in the liver are activated by HCV infection and produce IFN which not only attracts lymphoid cells to the infected area but also inhibits viral replication directly (reviewed in Lau 1994, Zhu, Zhao et al. 2003, Guidotti and Chisari 2006, Dustin and Rice 2007).

1.7.2 Interferon (IFN) system

Interferons (IFNs) are a group of secreted cytokines which together constitute the interferon induced innate immune response. They have antiviral, anti-proliferative and immunomodulatory functions. They are classified into type I, II and III IFNs depending upon their different amino acid sequences. Type I interferon further constitutes several types of which α and β IFN genes are mainly induced in response to viruses. For this reason instead of type I IFN the term α/ β IFNs is more often used in relation to response to viruses (Randall and Goodbourn 2008). Type I Interferons are derived from intronless genes found on the short arm of human chromosome 9 (9.21p3) and they share the same receptor and have overlapping functions (Brideau-Andersen, Huang et al. 2007, Randall and Goodbourn 2008). Multiple IFN α subtypes have been found in mammalian species. IFN α subtypes have approximately 80% amino acid sequence similarity (Randall and Goodbourn 2008). Type 1 IFNs are produced by a broad range of cells in the body and induce an antiviral state in virus infected cells and surrounding tissue by setting in motion a complex signalling pathway that results in transcription of

hundreds of ISGs which have strong antiviral activity (reviewed in Katze, He et al. 2002, Biron 2001).

Type III IFNs were recently described and consist of IFN λ 1 (IL-29), λ 2 (IL-28A) and λ 3 (IL-28B) (Meager, Visvalingam et al. 2005, Zhou, Hamming et al. 2007). Their receptors have mainly an epithelial distribution (reviewed in Borden, Sen et al. 2007). These are also induced in direct response to viruses and induce a similar group of antiviral genes as IFNs α/β (Onoguchi, Yoneyama et al. 2007). Type II IFNs are also called IFN- γ and they are not secreted in direct response to viruses but are produced by the subsets of T cells and NK cells in response to viral infection (Randall and Goodbourn 2008). IFNs α/β act on heterodimeric receptors broadly distributed in tissues to activate a signal transduction pathway which results in transcription of ISGs (Valarcher, Furze et al. 2003, reviewed in Galligan, Murooka et al. 2006). These genes are also induced directly by viral infection but the response is less effective compared to that induced by IFNs (reviewed in Randall and Goodbourn 2008). Type III IFNs are known to elicit similar response as that induced by Type I IFNs but their receptors are not widely distributed and their exact role in the antiviral response is under investigation (Meager, Visvalingam et al. 2005, Mennechet and Uze 2006, Zhou, Hamming et al. 2007). Marukian *et al*, has shown in a recent study that HCV infection of human foetal liver cells led to induction of type III IFN and ISGs suggesting that type III IFN may play a very important role in inducing innate immune response to HCV in liver cells (Marukian, Andrus et al. 2011).

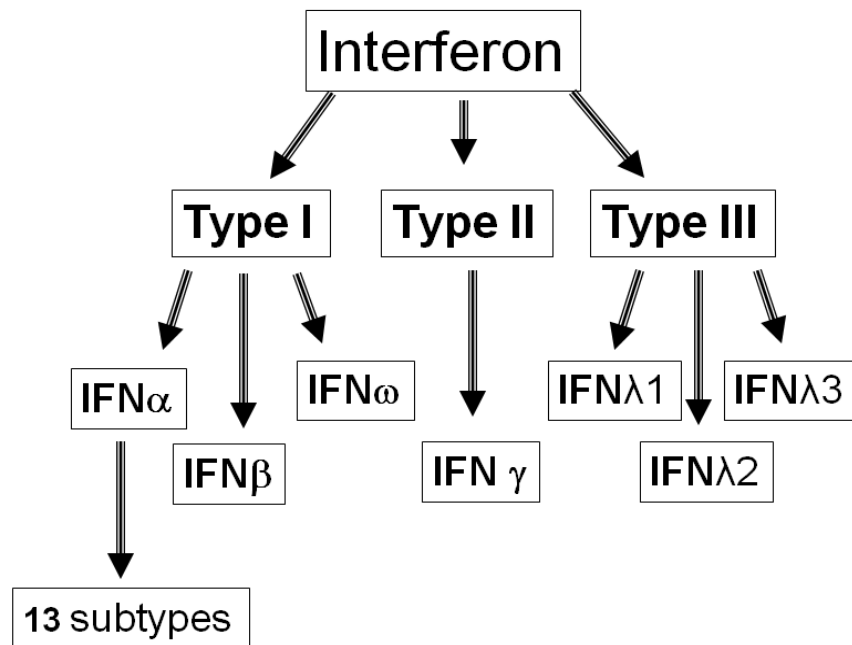


Figure 1.4: Different types and sub-types of Interferon

1.7.2.1. Induction of interferons (IFNs)

Type 1 interferon can be induced by Toll like receptor (TLR) dependent and TLR independent pathways. TLR dependent pathways are present primarily in cells of the myeloid lineage such as antigen presenting cells (APC) – including plasmacytoid dendritic cells and myeloid dendritic cells. This induction pathway involves TLRs which recognize and bind to the motifs present on different pathogens and leads to a wide variety of inflammatory responses as well as activation of dendritic cells and generation of specific T cell responses (Yonkers, Rodriguez et al. 2007). A number of viral motifs, including double stranded RNA (dsRNA), GU rich single stranded RNA (ssRNA) or endosomal DNA interact with TLRs (specifically TLR3, TLR7 and TLR9 respectively) which are found either extracellularly or intracellularly

(reviewed in Randall and Goodbourn 2008). This interaction leads to activation of a signal transduction pathway involving nuclear factor κ B (NF κ B) and/or interferon regulatory factor 3 (IRF3) and leads to production of IFNs and other pro-inflammatory cytokines. An alternative pathway for IFN induction involves the TLR-independent pathway that is activated after cytoplasmic molecules interact with viral RNA (chiefly replicating, dsRNA). This pathway may be more ubiquitous than the TLR induction pathways and is probably important in non-myeloid cells (reviewed in Randall and Goodbourn 2008). The RNA recognition molecules are RNA helicases variously named melanoma differentiation-associated gene 5 (*mda-5*) or retinoid-inducible gene I (*RIG-I*) (Staehele and Haller 1987, Zhou, Hassel et al. 1993). dsRNA and 5'triphosphate RNA produced by both DNA and RNA viruses are known to induce IFNs by binding to these intracellular detectors, (Mda5 and RIG-I) (reviewed in Marcus and Sekellick 1977, Marcus 1983) as shown in figure 1.5.

The downstream events that follow the activation of interferon inducers are less well established but activation of NF κ B and IRF-3 have a role in induction of IFN- β (Honda and Taniguchi 2006). Upon receipt of appropriate signals IRF-3 and NF κ B are translocated to the nucleus and bind to the IFN- β promoter leading to induction of IFN- β (Murray 2007). The mechanism of induction of IFN- α is less well defined (Meurs, Chong et al. 1990). It is thought that production of primary IFN i.e. IFN- β may lead to the induction of other transcription factors i.e. IRF-1, IRF-7 and IRF-9 which play a role in the induction of secondary IFN genes i.e. IFN- α genes (Morin, Braganca et al. 2002, Cebulla, Miller et al. 1999). In the context of HCV infection in

hepatocytes it is thought that TLR-independent pathway is induced after an HCV specific PAMP is recognised by the host receptor, specifically RIG-I, which leads to the production of IRF-3 which in turn mediates IFN and interferon stimulated genes (ISGs) induction and induces an innate antiviral defence response within days of HCV exposure limiting host cell permissiveness. RIG-I recognises blunted end dsRNA or ssRNA rich in polyuridine runs or bearing 5' triphosphates (Kato, Takahasi et al. 2011). Although RIG-I in Huh-7 cells recognises PAMP and responds to HCV RNA, Huh-7.5 cell line, which is a derivative of Huh-7 cell line, has defective RIG-I signalling which leads to blunted innate immune response, part of the reason why this cell line is so permissive to HCV infection in culture. In one study it was found that restoration of RIG-I signalling rendered Huh-7.5 cells non-permissive to HCV infection (Sumpter, Loo et al. 2005).

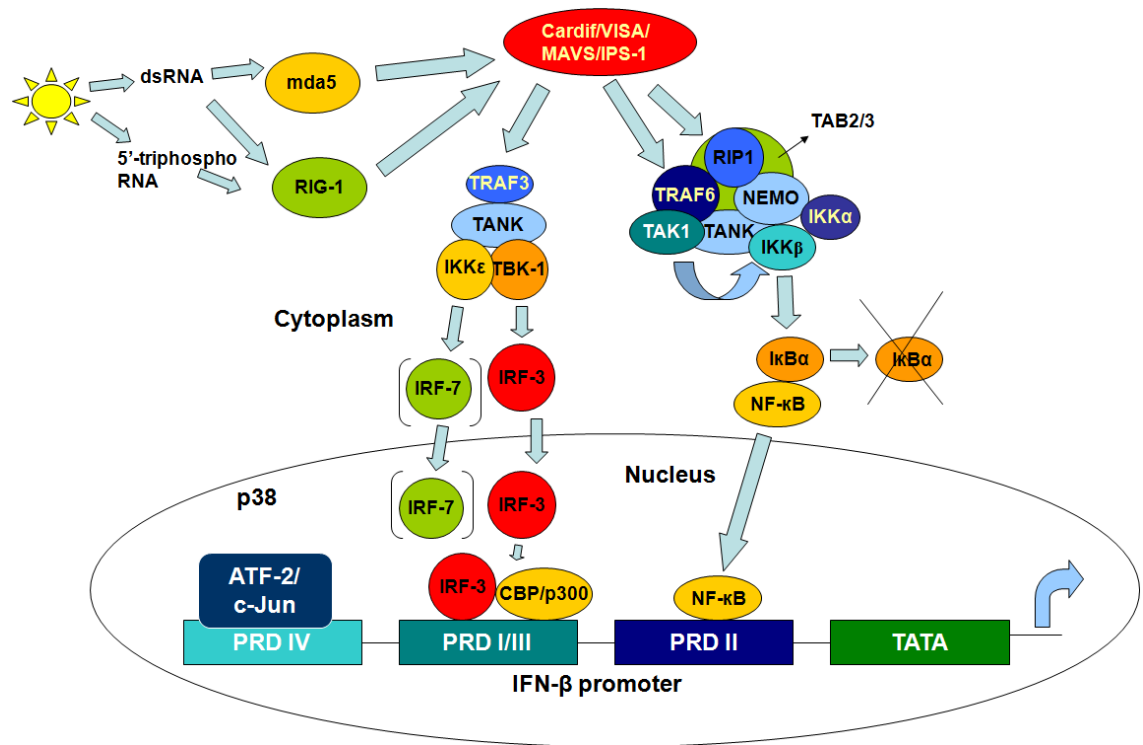


Figure 1.5: Mda5 and RIG-I dependent pathway for IFN- β induction (TLR-independent pathway): Viral dsRNA activates both mda5 and RIG-I and their specific domains recruit Cardif/VISA/MAVS/IPS-1, which in turn recruits signalling molecules which feed into NF κ B and IRF-3 pathway and thus lead to IFN- β production.

1.7.2.2. Interferon induced signalling

Pioneering studies using mutagenised cells that were selected for the loss of the cellular response to interferon have confirmed the pivotal role of the JAK-STAT signal transduction pathway in the cellular response to type I IFN. The JAK-STAT pathway is comprised of Janus kinases (JAK, a tyrosine kinase which is recruited to many receptor types after binding of ligands) and signal transducer and activators of transcription (STATs) (Stark, Kerr et al. 1998, Plataniias 2005, Stark 2007). STATs are phosphorylated by JAKs and bind to

DNA (Murray 2007). IFNs α/β binds to their heterodimeric cognate receptor composed of IFNAR1 and IFNAR2. IFNAR1 is linked to tyrosine kinase 2 (Tyk2) while IFNAR2 is linked to JAK1. Following binding of IFN to the receptor the JAKs are phosphorylated and then bind both STAT1 and STAT2 which in turn are phosphorylated by JAK1 and Tyk2 respectively. On phosphorylation STAT1 and STAT2 dimerise and this complex then binds to IRF-9 before translocating into the nucleus where it binds with Interferon stimulated response elements (ISRE) which are present in the promoters of all ISGs. The above signal transduction leads to the activation of ISGs which create the antiviral state in the cell (figure 1.6). Similar signalling is observed in response to IFN II in which IFN- γ binds to its cognate receptors leading to phosphorylation of STAT1 and its homodimerization to form gamma-activated factor (GAF) complex which activates the transcription of IFN- γ responsive genes (reviewed in Randall and Goodbourn 2008, Platanias 2005), Murray 2007, Gunji, Kato et al. 1994).

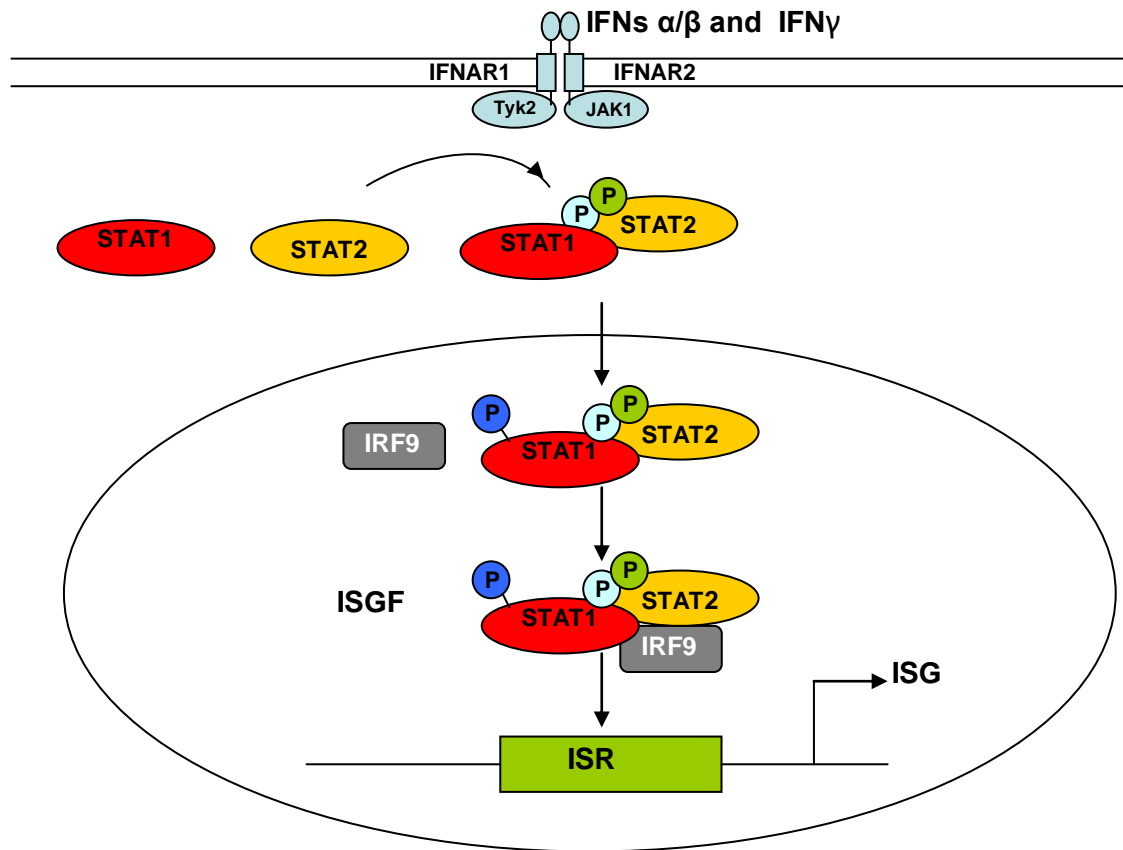


Figure 1.6: Signalling pathway activated by IFNs α/β leading to the production of ISGs: phosphorylated JAKs phosphorylate/dimerise STAT1 and STAT2 into a complex which binds with IRF-9 and translocates into the nucleus to produce ISGs.

1.7.3. Interferon stimulated genes (ISGs)

ISGs are the proteins which are induced by IFNs, many of which have known antiviral activity. There are hundreds of different ISGs and the function of the majority is unknown. The most well studied examples include; protein kinase R (PKR), myxovirus resistance protein A (MxA) GTPases and group of 2'5' oligoadenylate synthetases (2'5' OAS).

1.7.3.1. Functions of antiviral interferon stimulated genes

A) Protein kinase R (PKR)

PKR is a dsRNA-dependent protein kinase. It is a 68Kd protein containing a serine/threonine kinase and a dsRNA binding domain. The protein is present at low levels in all cells and levels increase after exposure to type I IFN. The kinase is generated in an inactive form but binding to conserved motifs in dsRNA leads to its activation which then phosphorylates the eukaryotic translation initiation factor 2 (eIF-2) and by doing so it prevents the recycling of initiation factors and hence halts the protein translation. In addition the kinase has a variety of other roles including promotion of apoptosis and activation of the NF κ B transcription factor which is needed for IFN β synthesis and which reduces viral spread to neighbouring cells (reviewed in Meurs, Chong et al. 1990, Gale and Katze 1998).

B) Myxovirus resistance protein A (MxA)

Myxovirus resistance proteins are IFN-inducible GTPases. MxA were originally discovered as IFN-induced proteins that are capable of blocking the replication of influenza virus. They interfere with viral replication by inhibiting the activity of the viral polymerase (Staeheli and Haller 1987).

C) 2'5' Oligoadenylate synthases (2'5'OAS)

2'5' Oligoadenylate synthases (2'5' OAS) are a family of enzymes that are activated by dsRNA to synthesize 2'5'-linked oligoadenylates. The 2'5' OAS

bind to an endoribonuclease L (RNase L). RNase L catalyses the cleavage of single stranded RNA resulting in the inhibition of protein synthesis. They cleave ribosomal RNA in a site-specific manner leading to the inhibition of protein synthesis (reviewed in Tan and Katze 2001, Zhou, Hassel et al. 1993) and help prevent viral replication.

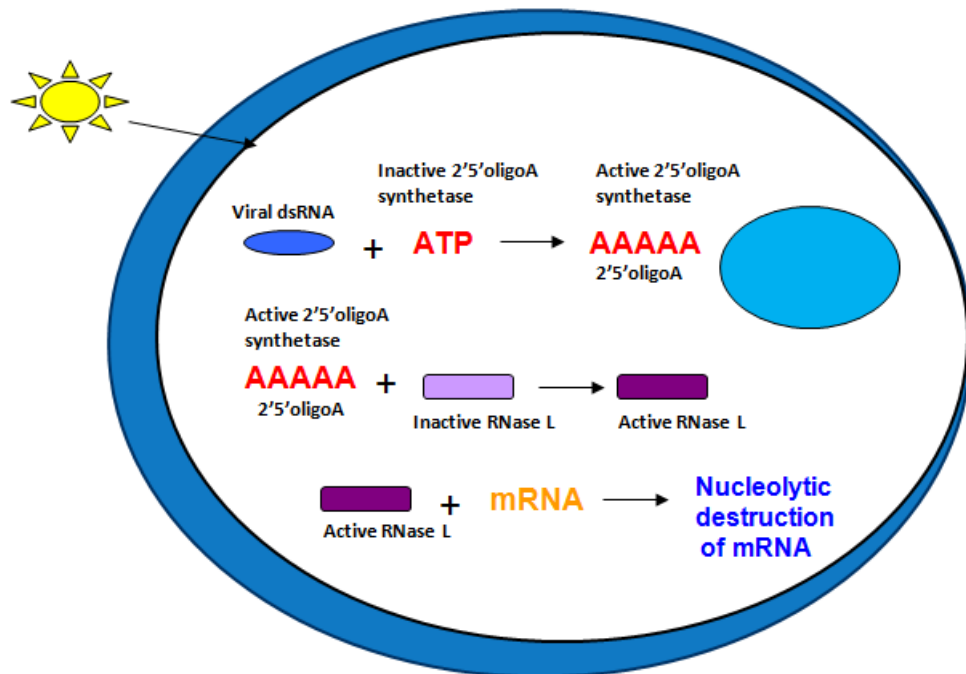


Figure 1.7: The mechanism of action of 2'5' OligoA synthetase: 2'5'OAS is activated on binding with viral dsRNA. Activated 2'5'OAS further activates RNase L which destroys viral mRNA and prevents the translation and replication of HCV.

1.7.4. Role of adaptive immune response to HCV infection

The adaptive immune response is acquired after birth and sets in motion either simultaneously or soon after the activation of the innate immune response to virus infection. It has a very important role in the control of

infection. B cells are involved in providing antibody mediated immunity against HCV while functional CD4+ and CD8+ T cells with antigen specificity are required for induction and maintenance of effective antiviral immunity (Heydtmann, Shields et al. 2001).

Antibodies produced in response to activation of B cells can act in different ways to provide immunity. They can bind to the pathogen and act as opsonins for antigen presentation or label infected cells to act as the substrate for the complement mediated killing. Alternatively, bound antibody can present infected cells to T cells, may directly block viral entry into the cells or bind to the virions and remove them from the circulation (Heydtmann, Shields et al. 2001).

CD4+T cells act as helper cells and provide effectors like IL-2, IL-4 and IFN- γ which mediate immune responses either by activating B cells and CD8+ T cells or by recruitment and activation of NK cells and macrophages (reviewed in Sen 2001). CD4+ T cells have a crucial role in activating CD8+ T cells and without these cells CD8+ T cells cannot assume their antiviral function appropriately. They also activate APCs and up-regulate MHC (major histocompatibility molecules) to promote antigen presentation to CD8+ T cells (reviewed in Guidotti and Chisari 2006, Randall and Goodbourn 2008, Brideau-Andersen, Huang et al. 2007, Onoguchi, Yoneyama et al. 2007). There are two ways by which CD8+ T cells act, either by lysis of the infected cell or by production of IFN- γ (reviewed in Doly, Civas et al. 1998) and TNF- α which controls viral replication directly by induction of antiviral state or indirectly by recruitment of cytolytic NK cells (reviewed in Guidotti and Chisari

2006, Randall and Goodbourn 2008, Brideau-Andersen, Huang et al. 2007, Onoguchi, Yoneyama et al. 2007.

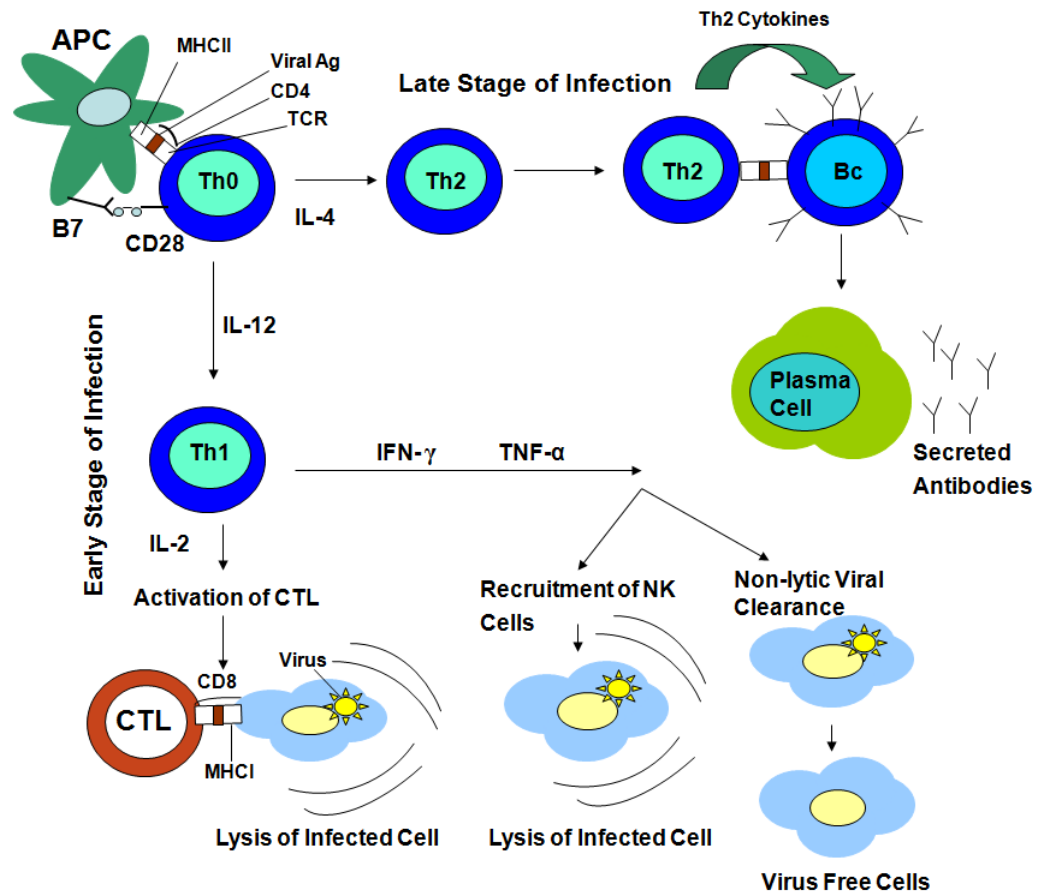


Figure 1.8: Adaptive immune responses to hepatitis C virus: Role of Th1 CD4+ T cells in activation of cytotoxic CD8+ T lymphocytes (CTL) and production of IFN- γ and TNF- α in the early stage of infection and Th2 CD4+ T cells in the production of antibodies in late stage of the disease.

Earlier studies have shown that patients who spontaneously clear their HCV infection show a wide specific and rapidly developing cellular immune response (Cooper, Erickson et al. 1999, Lechner, Wong et al. 2000), while in chronic HCV infection CD8+ T cells in peripheral blood are relatively low in

frequency (He, Rehermann et al. 1999). Similarly the CD4 T cell response is more efficient in those who clear HCV associated viremia (Gerlach, Diepolder et al. 1999). Sustained help from CD4+ T cells is required by CD8+ T cells, presumably in order to keep pace with viral replication and mutation (Lauer, Barnes et al. 2004, Burton, Klarquist et al. 2008). In order to resolve HCV infection rapid CD8+ T cells responses targeting multiple epitopes are pivotal. In chronic infection T cell responses as seen in the acute stage but are lost leading to a decrease in quantity and function of CD8+ T cells (Cox, Mosbruger et al. 2005). It is evident that in chronically infected patients despite persistent viremia and HCV sequence variation, new epitope specificity does not develop, as is seen in acute, self limiting infection. In cases where there are abundant CD8+ T cells and the patient slips into chronic infection, it is observed that these T cells are defective in function and could be referred to as 'stunned T cells'. Stunned T cells have limited ability to produce IFN, low proliferation rate and lose their lytic function (Cox, Mosbruger et al. 2005).

1.7.5. Effect of parainfluenza virus 5 (PIV5) on interferon induced antiviral state

Many viruses encode proteins that inhibit both the production of and the response to interferons by modulating the interferon signalling pathway and inhibiting cellular proteins participating in this cascade. They can interfere with components participating in the expression of ISGs including STAT1, STAT2, JAK1, or TYK2 (Cebulla, Miller et al. 1999). Here we illustrate this with reference to the PIV5 protein.

1. Structure of parainfluenza virus 5 (PIV5)

PIV5 previously known as simian virus 5 (SV5) is a non-segmental, single stranded and enveloped, negative sense RNA virus (reviewed in Goodbourn and Randall 2009) belonging to genus *Rubulavirus* of family *Paramyxoviridae* (Chatziandreu, Stock et al. 2004). In PIV5 a P gene encodes P and V proteins which have unique C-terminal domains but the same N-terminal domains. The V mRNA is a faithful transcript of the P gene while P mRNA has two non-template G residues in its transcript that alter its reading frame (Andrejeva, Young et al. 2002, Young, Didcock et al. 2000). The V mRNA is implicated in degradation of STAT1.

2. Mechanisms by which PIV5 circumvents interferon responses

A. Targeting signal transducer and activator of transcription 1

As discussed previously in section 1.7.3.2 STAT1 and STAT2 are critical for the IFN induced antiviral state as they are the transcription factors which transduce IFN signalling. The V protein of PIV5 forms either direct or indirect complexes with STAT1 and is known to block IFN signalling by targeting STAT1 for proteasome-mediated degradation (Young, Didcock et al. 2000, Carlos, Young et al. 2009). PIV5 infected cells are known to be defective in both phosphorylated and non-phosphorylated forms of STAT1 and formation of ISGF3 complexes is largely hampered by their absence (Andrejeva, Young et al. 2002, Precious, Young et al. 2005). This helps the virus to block both

IFN I and IFN II signalling and circumvent the immune responses of the host. Blocking IFN signalling prevents the appropriate antiviral response to be generated by the host. PIV5 infected cells do not show IFN induced responses and the production of ISGs is hampered (Carlos, Young et al. 2007).

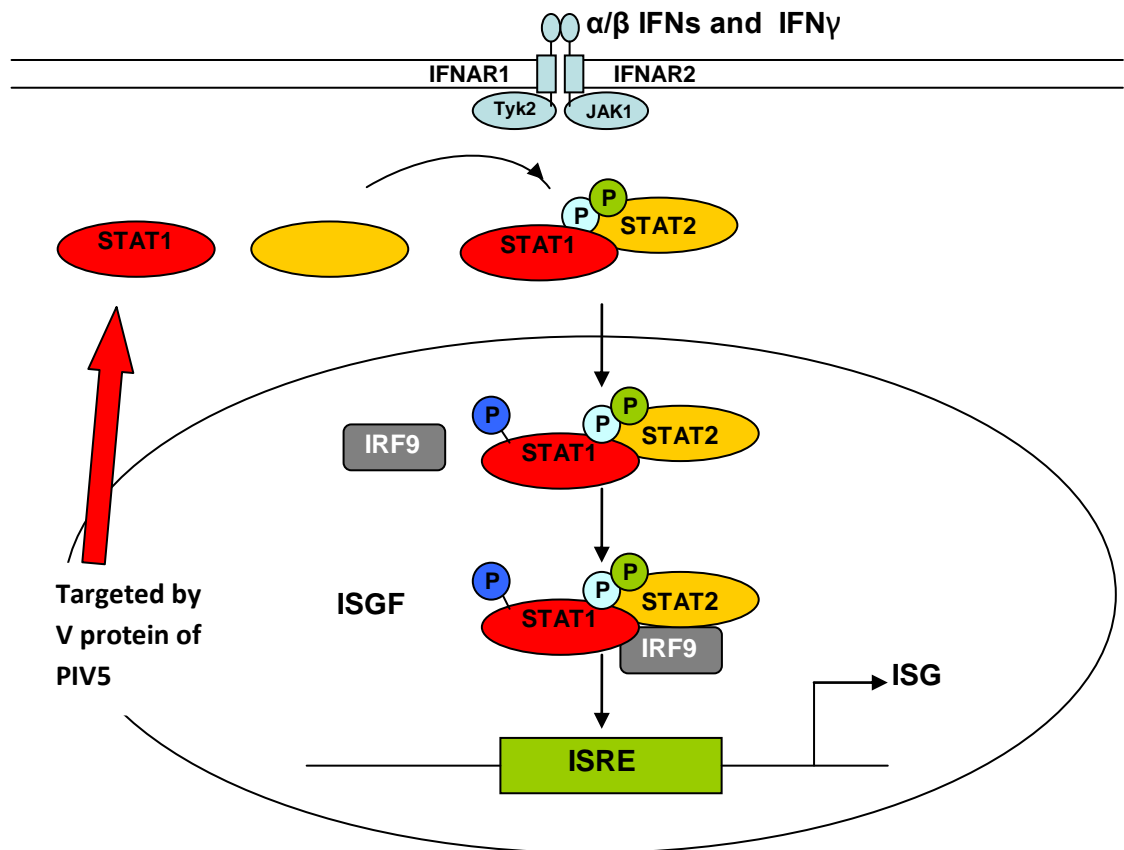


Figure 1.9: PIV5 targeting STAT1 for proteasomal-mediated degradation: Inhibition of signalling pathway by PIV5 V protein by targeting STAT1 and preventing the activation and production of ISGs.

B. Targeting melanoma differentiation associated gene 5

It is also thought that the V protein of PIV5 binds with and inhibits the action of IFN-inducible RNA helicase, called melanoma differentiation-associated gene 5 (*mda-5*) that recognises dsRNA and is an activator of IFN-induction cascade (Young, Didcock et al. 2000). It has also been debated whether PIV5-V does not degrade *mda-5* but inhibits the helicase domain of *mda-5* and prevents binding of dsRNA with *mda-5* and prevents its activation and hence the IFN induction (Andrejeva, Childs et al. 2004, Childs, Andrejeva et al. 2009).

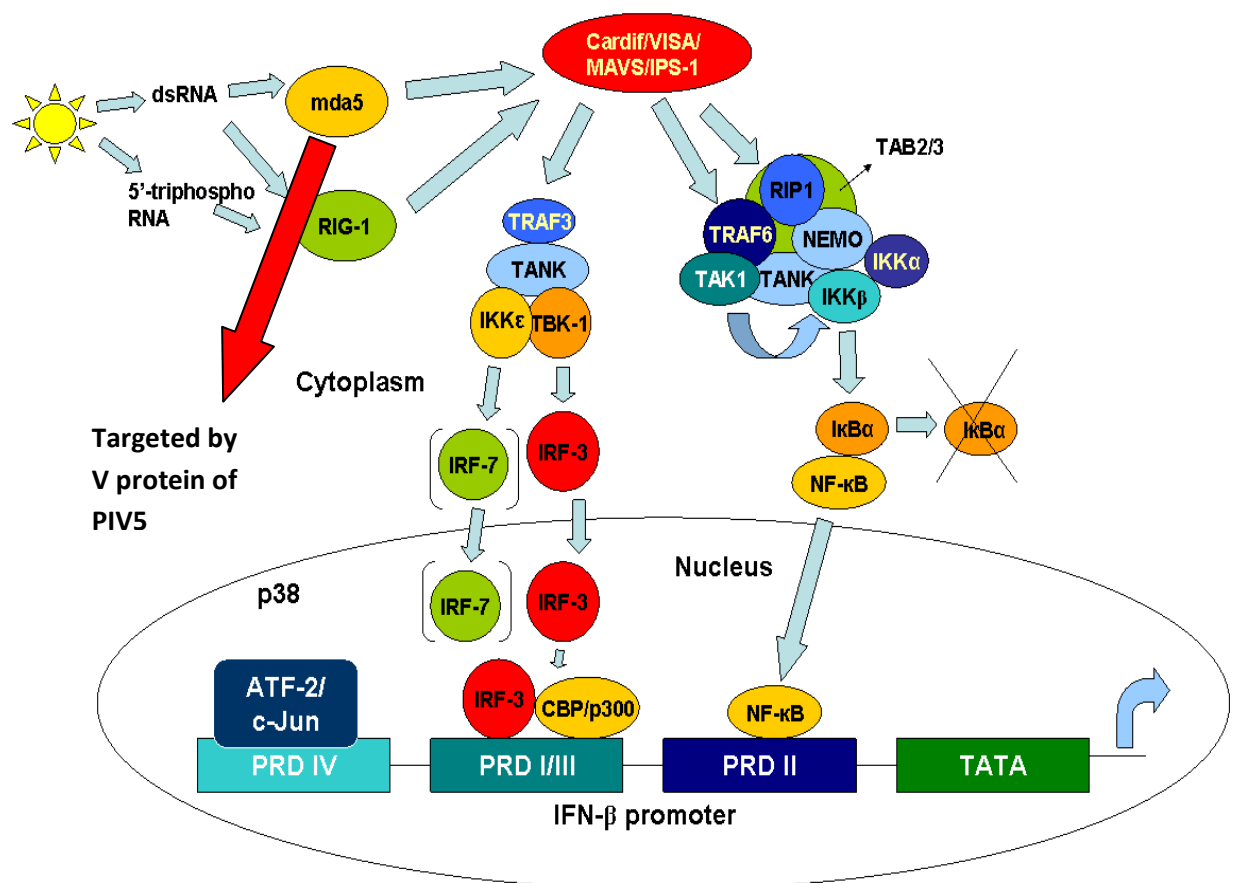


Figure 1.10: Inhibition of IFN Induction pathway by PIV5: Mda-5 is targeted by V protein of PIV5 and inhibits the signalling of mda-5 and

RIG-I dependent pathway for the IFN- β induction. Viral dsRNA activates both mda-5 and RIG-I which have domains that recruit Cardif/VISA/MAVS/IPS-1 which in turn recruits signalling molecules which feed into the NF κ B and IRF-3 pathways and lead to the IFN- β production.

1.7.6. Evasion of host immune responses

As discussed above HCV has the potential to cause both acute and chronic infections, however the exact mechanism of its host immune response modulation is unclear. The unusual ability of HCV to establish a persistent infection may, at least in part, be attributed to its potential to counteract host antiviral immune responses particularly the interferon induced defences and the cell-mediated acquired immune responses. These properties of the virus enable it to maintain chronic infectivity in the hepatitis C patients in 50-80% of infections.

Many viruses have developed mechanisms to block the IFN induced pathways at different levels; (Cebulla, Miller et al. 1999) by disruption of dsRNA and IFN receptor/JAK-STAT signalling events, inhibition of NF κ B functions and by various other mechanisms (reviewed in Levy and Garcia-Sastre 2001, Cebulla, Miller et al. 1999). For example, parainfluenza virus 5 (previously called simian virus 5) is known to inhibit immune responses by degrading STAT1 and inactivating mda-5 (Precious, Carlos et al. 2007) (described in detail above) and dengue virus is known to act on STAT2 and inhibits interferon signalling by degrading it (Ashour, Morrison et al. 2010). Some viruses inhibit ISGs for example influenza virus, herpes simplex virus,

epstein bar virus and HIV have developed mechanisms to inhibit PKR (Kaufman 1999, Cai, Carpick et al. 2000, reviewed in Katze, He et al. 2002). It is therefore not surprising to find that HCV has been implicated in immune system invasion at numerous levels.

1.7.6.1. Evasion of innate immune responses by HCV

The relation between Hepatitis C and interferon (IFN) has been extensively studied (reviewed in Tan and Katze 2001, He and Katze 2002, Taylor 2001). This is due to its use as therapy against HCV and its intrinsic role as a critical component of the host innate immune response. The interaction between HCV and IFN is difficult to evaluate as there are very few animal and tissue culture models that allow replication of HCV (Bartenschlager and Lohmann 2001, Gale and Beard 2001). Resistance of HCV to IFN (defined as persistence of HCV RNA in patient's serum after treatment) (Pawlotsky 2000) clearly plays an important role in determining the outcome of therapy, thus understanding the mechanisms underlying resistance will be of great importance in improving responses to therapy.

Different HCV viral genotype isolates show different levels of sensitivity to IFN with genotypes 2 and 3 being more responsive clinically. However, it is unclear why these genotypes are more responsive and the lack of a tissue culture model for the different viral genotypes precludes a formal analysis of viral sensitivity to IFN. For patients with genotype 1 HCV some patients/viruses appear to be more sensitive to therapy than others. Some of these differences may be due to differences in the host response perhaps mediated by IL-28 genotypes but viral variation may also play a role. Some

studies have shown that the NS5A region of HCV contains a region – the Interferon sensitivity determining region (ISDR) which confers IFN resistance, perhaps by interfering with dsRNA activation of PKR. However, not all studies support the association between ISDR sequence and the response to antiviral therapy (reviewed in Kittlesen, Chianese-Bullock et al. 2000, Wertheimer, Miner et al. 2003).

HCV is also implicated in blocking IFN through interfering with the IFN production pathway and attenuating the ISG expression and function. HCV infected cells show reduced production of type I IFNs and the NS3/4A protease of HCV is believed to play a major role in this phenomenon as blocking NS3/4A with an active site inhibitor leads to the restoration of RIG-I function and the IFN production in cells infected with HCV (Tasaka, Sakamoto et al. 2007). In few studies NS3/4A was shown to interfere with IRF-3 (activated by RIG-I) accumulation secondary to Sendai viral infection (Breiman, Grandvaux et al. 2005, Karayiannis 2005) and this protease is believed to degrade this molecule. As noted previously RIG-I is involved in the signal transduction cascade which leads to the IFN production and in a hepatoma cell line HCV expression was unable to transduce pathway involved in IFN production through RIG-I (Karayiannis 2005). The destruction of RIG-I by the HCV protease may play an important role in establishing chronic HCV infection and helps explain the mechanism of protease inhibitors in controlling infection.

HCV circulates in plasma in association with lipoproteins and resembles low density lipoprotein (LDL) particles. This phenomenon could disguise HCV

from the host immunity (Syed, Amako et al. 2010). Moreover, HCV replicates in host cells within a membranous web which might compartmentalise HCV from cellular nucleases, proteinases and RLRs (RIG-I like receptors) (Gosert, Egger et al. 2003).

Studies suggest that patients who do not respond to PEG-IFN and ribavirin therapy have an up-regulation of ISGs before therapy compared to those who respond to therapy. In one study, chimpanzees who had chronic HCV infection already had ISGs induced maximally and there was no further increase in ISGs after IFN therapy (Lanford, Guerra et al. 2007). In contrast, patients who respond to therapy show an impressive up-regulation of the ISGs after treatment (Honda, Sakai et al. 2010). The reason why this is the case is still an enigma but perhaps host induction of ISGs forces the development of viral variants that evade type I interferons.

1.7.6.2. Evasion of acquired immune responses by HCV

Persistence of HCV infection has been linked with the impairment of the acquired immune response (Takaki, Wiese et al. 2000). A number of different ways by which HCV kinetics outpace the acquired immune system have been described and these are outlined below.

A) Immune escape variants evade anti-HCV antibodies

Chronic infection with HCV is associated with the production of anti-HCV antibodies. One possible explanation of why HCV persists despite these antibodies is the heterogeneity in the HCV genome produced due to the

development of mutations at a very high rate. These mutations are due to the infidelity of the NS5B, the RdRp which during viral replication causes mutations in the HCV genome. These mutations accumulate successively resulting in the emergence of the quasispecies (different but related viral genomes) populations (Bukh, Miller et al. 1995) so that the virus has considerable heterogeneity and therefore it is hypothesised that the restricted antibody repertoire is unable to neutralise all viral variants in the quasispecies gene pool. Another explanation is that under antiviral pressures from the host, HCV undergoes adaptive mutations in the envelope gene (HVR-1 in E2) thereby constantly changing the antibody binding domain and preventing effective neutralisation of the viral antigen.

High variability in viral particles is due to the combination of several factors. It is partly due to the lack of proof reading activity of viral RNA polymerase (reviewed in Moradpour, Cerny et al. 2001), as well as co-evolution of HCV along with human populations for millions of years (reviewed in Pybus, Charleston et al. 2001) and due to the very high viral replication rates and rapid viral life cycle resulting in billions of viral particles produced every day in the HCV infected patients (reviewed in Neumann, Lam et al. 1998).

A number of other mechanisms have been implicated in HCV's evasion of humoral immune responses. These include HCV bound with lipoprotein while circulating in host blood mimicing the host molecules and this might protect it from host antibodies. HCV glycoproteins have been shown to interact with the high density lipoproteins (HDLs) and SR-BI in order to enter the host cell and thereby may evade neutralising antibodies (Logvinoff, Major et al. 2004).

Moreover, E2 glycans present on the surface of HCV modulate the viral entry and non-neutralising antibodies binding or direct cell-cell transfer of HCV may be implicated in avoidance of antibody neutralisation (Falkowska, Kajumo et al. 2007, Zhang, Wu et al. 2007, Brimacombe, Grove et al. 2011).

B) Impaired immune effectors

The immune response to HCV is polygenic with enormous expansion of T cells, as is the case with most pathogens. Given this huge expansion and mutation of the host response it seems unlikely that viral mutation in a single epitope is the only mechanism involved in the persistence of HCV infection. This raises the argument that there are mechanisms other than the evolution of immune escape variants which are implicated in the chronicity of hepatitis C infection (Eisen-Vandervelde, Yao et al. 2004).

Acute resolving HCV infection is associated with a rapid immune response leading to the generation of immune subsets i.e. CD4+ T cells and CD8+T cells which target multiple viral epitopes (Wertheimer, Miner et al. 2003). Conversely, in chronic infection this response is not seen and comparisons show that in chronic infection there was a delay in the formation of antibodies, markedly decreased IFN- γ production and lack of rapid expansion of immune subsets which only targeted a few epitopes and were insufficient to eradicate HCV (Takaki, Wiese et al. 2000). These hypotheses are supported by the studies done by Wertheimer et al (2003) who showed that people who recovered from the acute HCV infection showed a wide CD4+ and CD8+ T-cell response to specific epitopes which was not present in those who were chronically infected (Wertheimer, Miner et al. 2003). T cell

epitopes on APCs specific to class I and II MHC molecules can get mutated leading to a defect in antigen processing and presentation, T cell activation, antibody production and viral clearance (Eckels, Wang et al. 2000). In order to escape a multicentre cellular immune response, it is thought that multiple epitopes need to be altered. In one study, CD4⁺ T cells were inhibited in chimpanzees (previously resolved HCV infection) prior to re-infection. It was seen that despite memory CD8⁺ T cells being present HCV infection persisted due to epitope escape mutations in the viral sequence (Grakoui, Shoukry et al. 2003). However, epitope substitution was not actively seen in chronic infection in one study which suggested that epitope escape is characteristic of early infection (Chang, Rehmann et al. 1997). It was found that the mutations in the T cell epitopes, caused by replacement with viral amino acids, was a median of 16.7 fold higher in patients with persistent infection compared to the mutations at other sites and led to an impaired T cell recognition (Burke and Cox 2010). In chronic hepatitis C cases where the T cell epitopes alteration doesn't affect antigen processing or presentation, other factors such as the alterations in T cell receptor (TCR) affecting their engagement could be imperative in the mutant selection (Wolfl, Rutebemberwa et al. 2008). CD8⁺T cells reside in the liver for many years and could contribute to the pathogenesis of progressive liver disease (Claassen, Janssen et al. 2013).

In one study, T cell inhibitory programmed death receptor-1 (PD-1) was up-regulated in those patients who were unable to clear HCV infection (Urbani, Amadei et al. 2006). Other reports have shown the presence of these receptors in CD8⁺ and CD4⁺ T cells. Blocking of signalling through PD-1

receptors led to the restoration of activated T cell function (Urbani, Amadei et al. 2006, Radziewicz, Ibegbu et al. 2007). However, in another study PD-1 expression on T cells was also seen in patients with resolved infection and there are speculations that PD-1 expression might not be responsible for HCV persistence on its own and may need other factors in order to contribute to chronic viremia (Kasprowicz, Schulze Zur Wiesch et al. 2008). In settings where PD-1 expressions were low despite high HCV RNA, T cell escape substitutions were seen. High PD-1 expressions were linked with antigen preservation in the chronic infection (Rutebemberwa, Ray et al. 2008). Hence PD-1 expression may play a role in the development of chronic infection but further studies will be needed to confirm or refute this and the availability of monoclonal antibodies targeting these receptors in humans may allow their function to be assessed.

Viral sequence mutations leading to formation of quasispecies, may also pose selection advantages by increasing viral replication and evading host immunity. Furthermore, viral proteins have been implicated in conferring resistance to host defences and in the development of immunomodulatory strategies against host defences. There is some evidence suggesting that the HCV core protein can interfere with the activation and expansion of T cells by interacting with T cells receptors (TCRs) directly. Various researchers have shown that the core protein can hinder the magnitude and frequency of T cell response (Kittlesen, Chianese-Bullock et al. 2000, Yao, Takasawa et al. 2001, Yao, Nguyen et al. 2001). Kittlesen et al (2000) studied the complement receptor gC1qR which is involved in the activation of the complement system and can be inhibited by the core protein directly

(Kittlesen, Chianese-Bullock et al. 2000, Yao, Takasawa et al. 2001, Yao, Nguyen et al. 2001). Nevertheless, studies have demonstrated the indirect effect of core protein in inhibiting the T cell activation and proliferation by modulating the cytokines secreted by macrophages and DCs to activate T cells (Large, Kittlesen et al. 1999, Lai, Gong et al. 2000, Bain and Inchauspe 2001, Sarobe, Lasarte et al. 2002, Soguero, Joo et al. 2002).

HCV E2 protein is considered as another viral component that may be responsible for causing immune dysfunction. It has been suggested that it may play a role in cross linking CD81 molecules and thereby reducing cellular activation (Tseng and Klimpel 2002, Crotta, Stilla et al. 2002). In one study cross linking CD81 molecules by monoclonal antibodies had the similar effect on NK cell function as that seen when cells were exposed to the E2 protein of HCV (Tseng and Klimpel 2002, Crotta, Stilla et al. 2002). It was further observed that E2 not only inhibited the cytotoxicity of NK cells but also attenuated the IFN production by NK cells in response to cytokines (Tseng and Klimpel 2002). DCs are antigen presenting cells which secrete cytokines like TNF- α /IL-12 (myeloid DCs) and IFN- α (plasmacytoid DCs) which derive CD4⁺ T cells towards Th1 and Th2 phenotypes. In one study, where DCs from healthy donor were stimulated with HCV core and E1 proteins it was seen that DCs became defective in orchestrating the T cell response (Sarobe, Lasarte et al. 2002). The data on the effects of different HCV proteins have, in the main, been developed by *in vitro* models in which HCV proteins have been presented to immunocytes under carefully regulated conditions. It is unclear whether these effects play a major role in chronic

HCV infection or whether such effects are laboratory artefacts of dubious significance.

A large body of evidence suggest that Immune responses can also be attenuated by the regulatory T cells (Tregs) in chronic HCV infection. CD4⁺CD25⁺ T cells were found to be in abundance in chronically infected individuals compared to those who resolved their infection. These cells not only prevent effective CD8⁺ T cell replication but also prevent secretion of IFN- γ from virus-specific CD8⁺ T cells (Sugimoto, Ikeda et al. 2003, Cabrera, Tu et al. 2004, Boettler, Spangenberg et al. 2005). Furthermore, studies on human tissue determine that CD4⁺FOXP3⁺ cells are the main type of Tregs present in the livers of HCV infected individuals and their activity may be protective against hepatic fibrosis (Ward, Fox et al. 2007, Sturm, Thelu et al. 2010).

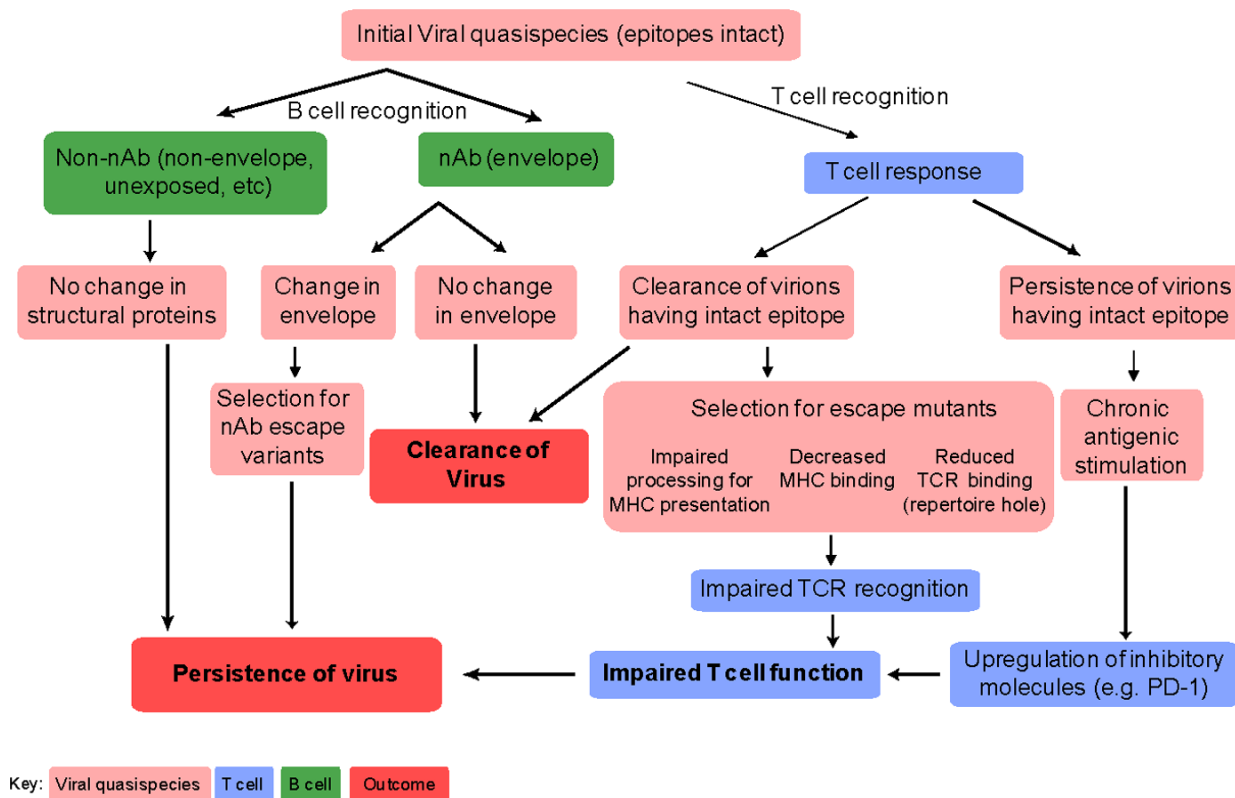


Figure 1.11: Viral evasion from the adaptive immune responses: the constraints placed on the virus due to the immune pressures of the host lead to the clearance or the selection for mutations that successfully evade the immune response. nAb = neutralizing antibody. TCR = T cell receptor (Burke and Cox 2010).

In summary, spontaneous or therapeutic HCV eradication requires an efficient and multi-centric immune response, while the chronicity of HCV is associated with a muted cellular immune response. The mechanisms underlying this immunological decline are yet to be fully elucidated. Multiple defects in the immune response secondary to HCV infection have been debated, some are described above. It is not evident which the most dominant phenomenon is or whether they all have equal part in the process.

1.8. Hepatitis C infection models

The lack of robust culture models for HCV has been a major hurdle in developing a full understanding of the virus and its replication. In the past decade there has been enormous progress in the development of various *in vitro* cell culture systems and *in vivo* small animal models. These model systems have furthered our knowledge of HCV infection, replication and have allowed preclinical testing of numerous novel antiviral compounds for the treatment of chronic HCV infection and are essential tools to develop more effective antiviral compounds. However these systems may be poorly representative of the viral replication in patients.

1.8.1. Cell based *in vitro* HCV systems

For years HCV researchers have strived for the development of a suitable cell culture system to study the viral replication, life cycle and host invasion and to test putative novel therapies. A number of groups tried to propagate HCV from patients' sera into primary cell lines from humans and chimpanzees with limited success (reviewed in Bartenschlager and Lohmann 2000) and earlier studies with HCV failed to generate replication competent viral clones. One explanation for this failure was the possibility that mutations occurring in the quasispecies gene pool rendered virus non-functional and this was enhanced during polymerase chain reaction amplification that led to the generation of molecular clones of HCV that were non-viable and could not be propagated in the *in vitro* cultures. However, development of so-called 'consensus genomes' with a master sequence representing the majority of

viral genomes circumvented this problem by terminating unsolicited, non-functional mutations. This led to the construction of a full-length functional clone of HCV complementary DNA from a genotype 1a strain (H77) and in 1997, this was shown to replicate in chimpanzee model. Hence, its RNA transcripts were found to be infectious (Kolykhalov, Agapov et al. 1997, Yanagi, Purcell et al. 1997). However, consensus genomes constructed successively thereafter from genotypes 1 and 2 did not effectively replicate *in vitro* (Bartenschlager and Sparacio 2007).

1.8.1.1. HCV (sub-) genomic replicon systems

Sub-genomic viral replicons for other flaviviruses, especially Yellow Fever were developed many years ago (Molenkamp, Kooi et al. 2003, Jones, Patkar et al. 2005). The success of this approach for a flavivirus, closely relating to HCV, led to attempts to generate an HCV replicon. Professor Bartenschlager's group was the first to successfully develop an HCV replicon from Con 1 strain (genotype 1b) by generating a bicistronic replicon construct engineered by replacing the structural protein-coding region of HCV along with P7 with a Neomycin phosphotransferase gene (Neo®) and encephalomyocarditis virus IRES element (EMCV IRES) (Lohmann, Korner et al. 1999). EMCV IRES promotes the translation of non-structural protein-coding sequences crucial for the viral replication. Upon transfection of RNA the constructs, manipulated in this way into a permissive cell line (Huh-7), drug-resistant (i.e. neomycin-resistant) cell colonies were isolated which had high levels of viral replication within them. These replicons self-amplified through synthesis of negative-strand intermediates. Subsequent analysis

confirmed that these HCV replicons were indeed capable of self-amplification through synthesis of a negative-strand replication intermediate and remained stable in cell culture for many years (Lohmann, Korner et al. 1999, Pietschmann, Lohmann et al. 2001).

A much more permissive cell line was generated by treating the replicon infected cell line Huh-7 with Interferon alpha (IFN α) and eliminating the virus. Retransfection of this cell line with replicon RNA resulted in much higher replication rates (reviewed in Krieger, Lohmann et al. 2001, Lohmann, Korner et al. 2001, Lohmann, Hoffmann et al. 2003, Blight, Kolykhalov et al. 2000, Blight, McKeating et al. 2003). The exact mechanism for the enhanced replication seen in Huh-7 derived cells is unknown (reviewed in Sumpter, Loo et al. 2005) but several such lines were later developed such as Huh-7.5 and Huh7-Lunet cells which were permissive to the viral replication (Blight, McKeating et al. 2002). Although the mechanism of the adaptation in Huh-7.5 that allows HCV replication to occur is unknown it seems probable that some significant change in gene expression has occurred that allow HCV to replicate in these cells. In the replicon itself adaptive mutations have been reported along the full length of the non-structural protein encoding region and the replicon depends upon these tissue-culture-adaptive mutations in order to replicate effectively in host cell lines. These mutations mostly cluster in the NS3 and NS5A regions. Although the mechanism by which these mutations enhance replication is largely unknown, these may modify the activity of viral replicases to enhance RNA replication or alter the phosphorylation state of the NS5A protein and affect interaction with VAP-A (vesicle-associated membrane protein-associated protein A) of the host

Krieger, Lohmann et al. 2001, Abe, Ikeda et al. 2007, Yi, Ma et al. 2007, Jones, Patkar et al. 2005, Liu, Ansari et al. 2006, Sarobe, Lasarte et al. 2002, Lohmann, Korner et al. 2001, Lohmann, Hoffmann et al. 2003, Blight, Kolykhalov et al. 2000, Blight, McKeating et al. 2003, Evans, Rice et al. 2004). VAP-A is considered to be a host factor vital for HCV replication and binds to both NS5A and NS5B. Novel antiviral compounds have been tested effectively on the replicon to inhibit the action of proteases like NS3 or the polymerase activity of NS5 (reviewed in Bartenschlager 2005) and many drugs have been successfully developed using this system.

There are a number of limitations of the replicon system which are due to the failure of the replicon to generate infectious virions and the presence of tissue culture adaptive mutations. The later problem is a source of great concern as these mutations are not found in naturally occurring isolates and when transferred into virus and used to infect chimpanzees the mutations showed an attenuated phenotype with defected viral packaging and secretion. The relevance of the replicon system to viral replication in humans is unclear but there are clear differences with uncertain effects (reviewed in Bukh, Pietschmann et al. 2002).

1.8.1.2. HCV pseudotype particle (HCVpp) system

To study HCV entry into cells, clearly not possible with replicons, a novel pseudotype particle system has been developed. The HCVpp system was constructed by packaging HCV envelope glycoproteins, E1 and E2 into a retrovirus and lentivirus core protein to study the mode of viral binding and entry into the cells (Bartosch, Dubuisson et al. 2003). The exact role of the

envelope proteins remain to be unravelled though evidence suggests that E2 possesses receptor-binding properties and establishes covalent binding with the viral entry receptors (Pileri, Uematsu et al. 1998, Bertaux and Dragic 2006). The infectious HCVpp has allowed the identification of many viral entry receptors such as CD81 receptor which is necessary but not entirely sufficient for HCV entry (Bartosch, Dubuisson et al. 2003, Cormier, Tsamis et al. 2004, Lindenbach, Evans et al. 2005, Wakita, Pietschmann et al. 2005, Zhong, Gastaminza et al. 2005) and needs the assistance of other receptors such as low density lipoprotein receptors, dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN), liver-specific intercellular adhesion molecule-3-grabbing non-integrin (L-SIGN), claudin-1, claudin-6, claudin-9 and scavenger receptor class B type1 (SR-BI) (Jia, Du et al. 2008, Regeard, Trotard et al. 2008, Stamataki, Grove et al. 2008, von Hahn and Rice 2008). Occludin was identified as another critical factor in the establishment of HCV entry into the host cells by the use of HCVpp system (Ploss, Evans et al. 2009). Moreover, E1 and E2 neutralizing agents prevented HCVpp entry into hepatoma cells (Hsu, Zhang et al. 2003, Bartosch, Dubuisson et al. 2003, Lagging, Meyer et al. 1998). Hence, HCVpp continues to provide understanding of HCV binding, attachment, and internalization processes.

1.8.1.3. Infectious HCV in cell-culture (HCVcc)

Studies of the release of infectious virion from the replicon system are not possible as the replicon does not contain the structural genes. Moreover, there has been limited success in attempts to generate full length replicons

that contain viral envelopes and release viral particles (Blight, McKeating et al. 2002, Pietschmann, Lohmann et al. 2002). In this context, the emergence of a genotype 2a consensus genome called JFH-1, isolated from a Japanese patient with fulminant hepatitis C, was a major turning point (Kato, Date et al. 2003). Replication of *in vitro* transcribed JFH-1 was 20-fold higher than non-JFH-1 replicon and it did not require adaptive mutations for replication (Kato, Date et al. 2003, Zhong, Gastaminza et al. 2005, Wakita, Pietschmann et al. 2005). HCVcc has enabled the study of full viral life cycle *in vitro*. Furthermore, this virus is able to infect Huh-7.5 cells directly. The *in vitro* transcribed full-length JFH-1 clone and a genetically engineered chimeric genome (another genotype 2a isolate- J6) both produce viral particles that are infectious in tissue cultured cells, in chimpanzees and in chimeric mice transplanted with human hepatocytes (reviewed in Friebe, Boudet et al. 2005, Evans, Rice et al. 2004, Lindenbach, Evans et al. 2005, Yi, Ma et al. 2007). Similarly, HCVcc replicating system from genotype 1a virus (H77-S) produces infectious virions in tissue culture on being transfected into the permissive Huh 7 cell line but is not as efficient as JFH-1 (Yi, Villanueva et al. 2006). It has also been possible to produce infectious particles in cell culture by Con 1 strain, but again it is not as efficient as JFH-1 and infection is marginal (Pietschmann, Zayas et al. 2009).

Since its discovery seven known HCV genotype envelopes have been engineered into JFH-1 but these inter-genotypic variants result in lower infectious virion production than wild-type JFH-1 (Yi, Ma et al. 2007, Pietschmann, Kaul et al. 2006, Gottwein, Scheel et al. 2009). These envelope hybrids do not contain replication enzymes from any strain other

than JFH-1 and although success in introducing protease enzymes from other genotypes has been achieved (like Jc1-pFK JFH1, J6/C-846 clones which contain proteases and NS4A coding sequences from the major genotypes) hybrid virions containing other replication enzymes have not yet been produced (Imhof and Simmonds 2010).

In summary, it seems probable that some viral proteins may be transferred successfully into replicons and JFH-1 and there have been recent successful attempts to generate JFH-1 virions which contain protease domains from other viral genotypes. However, to-date it has proven difficult to generate replicons or JFH-1 chimeras containing multiple domains from different genotypes. This has adversely affected study of combination antiviral therapy (Imhof and Simmonds 2010). A number of groups have managed to create JFH-1 clones with protease or NS5A or NS5B domains from other viruses (Li, Ramirez et al. 2012). They have, however not been able to transfer two domains so they cannot study combination therapy – hence HCV replicating model with heterogeneous patient genotypes is critical in the new direct acting anti-viral combination era.

1.8.1.4. Primary human hepatocytes (PHHs) cell culture

The lack of efficient HCV propagation in PHHs through sera of infected patients has been a major hurdle in the development of *in vitro* culture systems for HCV. This could be attributed to the loss of differentiated phenotype, minimum cell division, lack of reproducibility and low level of HCV replication in hepatocytes outside human body. HCV specific antibodies in the serum of infected patients might be the cause of impaired replication *in*

vitro (reviewed in Gondeau, Pichard-Garcia et al. 2009, Bartenschlager and Lohmann 2001). Moreover, it is well known that the liver has a highly specialised and heterogeneous structure and the *in vitro* systems with similar level of complexity have proven problematic.

Primary human hepatocytes (PHH) are closest to the physiological liver cells *in vivo*. However, it has not been possible to infect PHH with serum derived HCV. Moreover, their life span decreases due to the loss of differentiation phenotype. Various groups have tried different techniques to increase the longevity of hepatocytes and to maintain their specific functionality by growing them with other cells to create co-cultures, by creating 3D environment for cell-to-cell interaction i.e.; matrigel embedded liver cell lines and by growing in the extracellular matrix etc (reviewed in Bhatia, Balis et al. 1999, Sivaraman, Leach et al. 2005, Khetani and Bhatia 2008). In one study, Ploss et al. have described a robust but relatively cumbersome technique using a stensil (microscale) to grow hepatocytes and fibroblasts in spheroids in order to maintain hepatocyte phenotype, polarity and cell-to-cell contact. This procedure was referred to as micropatterned co-culture technique. It was used to demonstrate all stages of HCV life cycle and could maintain HCV infection for weeks (Ploss, Khetani et al. 2010). Complex polarity of hepatocytes could not be maintained within most *in vitro* models. However, co-cultured primary human hepatocytes exhibit polarised membranes with HCV entry factors localised at sites similar to human liver cells. These co-cultures generated proto-bile canalicules suggestive of cellular polarity and hence, maintain HCV replication for longer (Ploss, Khetani et al. 2010). The co-culture model could be used as a platform to test antiviral therapeutics.

However, this system is not widely available and has not been replicated outside the inventor's laboratory.

Many groups have tried using other cell types which resemble the *in vivo* phenotypic, function and physiology of hepatocytes. The hepatocytes derived from pluripotent stem-cells were used successfully to recapitulate hepatocyte biology, metabolic activity and non-transformed differentiated phenotype. These cells were engineered to mimic hepatocytes and were either obtained from embryonic cell line or induced by reprogramming with various agents. Various phases of HCV life cycle have been successfully studied using this approach. These cells bear receptors for HCV entry process, demonstrate HCV replication and possess ability to release progeny infectious viral particles (Yoshida, Takayama et al. 2011, Roelandt, Obeid et al. 2012, Si-Tayeb, Duclos-Vallee et al. 2012, Wu, Robotham et al. 2012). In one study, infection with the virus led to the release of ISGs (reviewed in Marukian, Andrus et al. 2011). Moreover, viral replication cessation was carried out by the use of specific inhibitors. Though not hepatocytes per se this cell line is the closest to the ideal model containing hepatocytes which retain their biology and is a major breakthrough in the field of hepatitis C. Important stages of HCV signalling pathways can be studied in a greater detail by using this system.

Non-transformed human foetal liver cells (HFLCs) were used in a few studies when HCV virions were secreted into the culture for almost 2 months after infection and the virus was also reported to induce interferon gene expression secondary to established HCV infection. Hence, HFLCs are effective prototypes of hepatocytes *in vivo* and induce HCV genome

expression *in vitro* (Lazaro, Chang et al. 2007, Andrus, Marukian et al. 2011). It was however noticed that different donor foetal cells expressed different types of ISGs which might not simulate the inflammatory response of *in vivo* hepatocytes (reviewed in Marukian, Andrus et al. 2011).

1.8.1.5. Immortalised hepatocytes and hepatoma cell lines

In the light of the above mentioned issues with using primary human hepatocytes *in vitro*, various research groups have generated immortalised liver cell lines such as PH5CH and HuS-E cells. Hepatocytes in these cell lines were immortalized by using T antigen of simian virus 40 and E6/E7 genes of the human papillomavirus (Ikeda, Sugiyama et al. 1998, Aly, Watashi et al. 2007). However, HCV replication was restricted in these cells and the production of viral particles was not observed, which makes them unsuitable for long term studies.

Certain hepatoma cell lines used to evaluate HCV life cycle have served as solid platforms for HCV research. As discussed above prolonged interferon- α treatment of Huh-7s (hepatoma cells) after infection with HCV sub-genomic replicon led to development of highly permissive cell lines. Re-transfection of clonal subpopulations with HCV replicon led to increased replication especially in one cellular clone which was referred as Huh-7.5 cells (Blight, McKeating et al. 2002).

Higher viral replication in Huh-7.5 cells is accounted by the defect in RIG-I pathway which is an important substrate in imparting immune response against HCV (Lanford, Guerra et al. 2003, Sumpter, Loo et al. 2005).

Furthermore, HCVpp and HCVcc were able to infect, replicate and release infectious particles in Huh-7 and related cell lines for considerably long time. These cell lines have contributed to the significant amount of knowledge into the biology of HCV. However, as these cells are derived from the human hepatocellular carcinoma they have reduced differentiation, abnormal proliferation, atypical gene regulation and deviant signalling pathways (Durantel and Zoulim 2007). Various groups have used different ways to reduce proliferation and improve differentiation of these cell lines. DMSO was reported to inhibit cell growth and enhance cytogenetic differentiation of hepatocytes by increasing differentiation markers along with supporting HCV infection (Sainz and Chisari 2006).

Hep3B is a hepatoma cell line which shows high level of HCV entry, but low level of replication (Sainz, Barretto et al. 2012). Huh-6 cell line derived from hepatoblastoma, expresses low levels of claudin-1, affecting the entry process of HCV (Haid, Windisch et al. 2010). In one series, the PLC/PRF/5 cell line taken from a primary liver carcinoma showed three fold higher HCVpp entry. However, this cell line did not support HCVcc infection in the same series (Blanchard, Belouzard et al. 2006, Benedicto, Molina-Jimenez et al. 2009). After comparing different hepatoma cell lines for different aspects of viral life cycle it was observed that Huh-7 cells were more supportive of HCV replication compared to other cell lines which supported HCVpp entry step, but were deficit in maintaining later stages of HCV life cycle (Sainz, Barretto et al. 2012). Many factors could be imperative in increasing the permissiveness of Huh-7 cells. It is speculated that other cell lines might have stronger immune response leading to reduced viral replication. Huh-7

cells are thought to express the microRNA-122 (miR-122) gene in higher proportions compared to the other cell lines. MiR-122 is a microRNA (miRNA) present in abundance in the liver tissue and it paradoxically increases HCV RNA replication (Jopling, Yi et al. 2005, Girard, Jacquemin et al. 2008). In order to increase our understanding of HCV life cycle it would be beneficial to explore other cell lines with greater permissiveness to HCV infection as well as examine factors which are responsible for increasing HCV replication.

1.8.1.6. Liver slices

Lagaye and colleagues (2012) used adult human liver tissue slices to study HCV infection in which they showed effective HCV infection and production of infectious particles. Liver tissue dissections were obtained from liver resections carried out because of the liver metastasis which did not have any underlying hepatic disease. Nonetheless, HCV entry into the liver slices was inhibited by using neutralising antibodies against CD81 and E2 protein in a dose dependent fashion (Lagaye, Shen et al. 2012). This model very closely resembles the hepatic microenvironment and dynamics of HCV infection in the hepatic parenchyma can be studied by using this model. Limited availability and short life of the liver tissue are two major drawbacks of using this assay.

1.8.2. The chimpanzee model for HCV infection

There are many features in chimpanzees which are similar to humans, most likely owing to the close genetic relationship. Chimpanzees are one of the

most recognized models for the study of hepatitis C. They played a critical role in the discovery of hepatitis C (Choo, Kuo et al. 1989) and continue to play a very important role in facilitating our knowledge of HCV infection. They have provided us with a platform for the study of natural history, morphology, transmission, genetic drift, factors involved in viral clearance, cloning of HCV nucleic acid, clinical course of infection, the role of immune responses, virus-host interaction, intrahepatic responses following infection, immunogenicity, efficacy of vaccine and immunopathogenesis (Prince and Brotman 1994), (Bukh, Kim et al. 1998, Walker 1997, Bukh 2004, Lanford, Bigger et al. 2001). Chimpanzees respond differently to drugs as compared to humans and mostly show close resemblance to non-responders to interferon and ribavirin therapy. In spite of this chimpanzees are important for pre-clinical drug testing and to study the pharmacological effects of drugs (Lanford, Guerra et al. 2006, Lanford, Guerra et al. 2007, Chen, He et al. 2007, Carroll, Ludmerer et al. 2009). Research on chimpanzees however is increasingly limited owing to the ethical constraints and economic issues. For this reason much attention has been focused on the development of small animal models.

1.8.3. Small animal models for HCV infection

Small animal models like rodents are easily maintained compared to chimpanzees because they are small, have short gestational period, are routinely used in experimental work, are readily available, are not in the category of endangered species, like chimpanzees, and are highly cost effective. Most of these rodent models have been rendered immunodeficient

by the genetic manipulations and numerous inbred strains can be produced to achieve these genetic and immune deficiencies. Furthermore, transgenic mice over-expressing HCV proteins have been developed and used over many years to study the effects on liver pathology (Kremsdorf and Brezillon 2007, Barth, Robinet et al. 2008). However, replication in these models requires complex manipulations including grafting of human hepatocytes and an environment of immune deficiency (Wu, Konishi et al. 2005).

Almost two decades ago a transgenic strain of mice over expressing the urokinase plasminogen activator (uPA) enzyme in hepatocytes was developed which exhibited severe liver toxicity (Heckel, Sandgren et al. 1990) and liver failure. The damaged liver could be repopulated with hepatocytes from mice, woodchucks or humans after rendering these mice immunodeficient (chimeric human liver uPA/SCID mouse model). These grafted cells were integrated in the murine liver and formed an organized structure (Meuleman, Libbrecht et al. 2005). Inoculating these models with serum from HCV infected donors showed high viral titres, replication was observed and infection lasted for months without hepatic damage (Meuleman, Libbrecht et al. 2005). In some models HCV replication was detected after inoculation with HCV positive patients' sera for 3 generations with the release of infectious particles (Mercer, Schiller et al. 2001). It was also noticed that the models containing hepatic cells from different donors exhibited different responses. This proved that host factors play an important role in overcoming viral infection (Walters, Joyce et al. 2006). This model is by far the closest in physiology to the human infection and has been useful for determining antiviral drug efficiency (Hiraga, Imamura et al. 2007,

Vanwolleghem, Meuleman et al. 2007, Kneteman, Howe et al. 2009). Some of the shortcomings of this model are that the immune deficiency required for the successful xeno-grafting restricts studies on the adaptive immune responses, the immunopathology and the development of vaccines. Additionally, generation of these chimeras is not easy because of the requirement of high quality donor hepatocytes. Furthermore, intrasplenic transplantation of fresh or frozen human liver cells was to be carried out within first 2 weeks of life leading to high mortality rates (Mercer, Schiller et al. 2001). The technical difficulties and high costs of this approach restrain its widespread use. Hence, this model is not widely accessible and is only available in a very small number of centres (Boonstra, van der Laan et al. 2009).

An alternative approach has been tried in a rat model of infection involving immunological tolerance to allow the generation of small animal models with functional immune cells. The principal underlying the development of an immunologically tolerized rat model was to expose foetal rat during the immune development stage inside the gravid uterus to human hepatoma cells so that the rat immune system would be tolerized to human hepatocytes and would not reject transplanted human liver cells after birth. HCV inoculated into the spleen of such mice showed 62% infectivity but viraemia remained low in this model. Later immune-mediated liver damage did eventually take place making this model suboptimal for antiviral drug testing (Wu, Konishi et al. 2005). Moreover, the benefit of functional rat immune cells was masked by their lack of genuine immune response as seen in humans. This muted immune response was due to the lack of recognition of HCV

antigens in the presence of human hepatocytes by the rat major histocompatibility complex.

Alternative mouse models with heterotopic human liver grafts have been created by implanting human liver fragments under the kidney capsule or behind the ear pinna of mice. However, they are subject to progressive loss of architecture because of lack of portal circulation and bile drainage (Galun, Burakova et al. 1995, Maeda, Watanabe et al. 2004). The loss of heterotopic liver graft is a major confounding factor in the interpretation of viraemia and low viral replication limits the utility of this model.

Dorner *et al*, attempted murine humanisation using genetic approach. In one study, two human genes required for HCV entry (CD81 and Occludin) were genetically engineered to create a transgenic mouse model. This model was used to recapitulate initial steps of HCV entry process. However, there was a loss of HCV signal after 72 hours indicating lack of suitable machinery required for HCV replication and strong antiviral immunity observed by increase in NK cells (Dorner, Horwitz et al. 2011).

1.9. Genomic diversity of HCV quasispecies

In an infected individual HCV is present as a heterogeneous mixture of closely related but genetically divergent variants, which are known as the 'quasispecies'. These variants continuously emerge due to accumulation of errors caused by the infidelity of RNA dependent RNA polymerase (RdRp) and rapid replication rate of HCV producing 10^{12} virions per day in an infected individual (reviewed in Pawlotsky 2003). Their emergence is

secondary to the survival response against the host immune system and the concomitant antiviral treatment and leads to mutant selection which replicate against unfavourable conditions in the host. Changes within the quasispecies could be reflected in the resistance to treatment (reviewed in Pawlotsky 2006).

Some regions of HCV genome are more prone to nucleotide mutation than others depending upon the viral protein function and specificity to the immune response. NS3, NS3 helicase, NS5A and NS5B are fairly conserved regions. HVR-1 present at the N-terminal end of E2 viral protein is the most variable region among quasispecies (reviewed in Pawlotsky 2006). This genetic heterogeneity is either spontaneous or is thought to be due to immune pressures from the host and neutralising antibodies leading to formation of mutants which escape the immune response (Bukh, Miller et al. 1995). Quasispecies differ from genotypes which show major genetic differences amongst them, whereas quasispecies represent minor genetic change. Also quasispecies continue to evolve over time, while genotypes remain stable.

Quasispecies analysis has not been directly introduced into HCV management. However, it is a major research target. Chronic HCV patients tend to have a greater diversity amongst the quasispecies variants compared to the patients with acute or self limiting infection (Farci, Shimoda et al. 2000). The kinetics of quasispecies emergence during acute infection may indicate the later course of infection. Quasispecies have epidemiological importance and can be used to link infection to its source (Gretch 1997).

According to one study, quasispecies variability decreased in responders of treatment with interferon and ribavirin compared to non responders, in which it either increased or remained stable (Puig-Basagoiti, Forns et al. 2005).

Quasispecies might vary within the same host among different tissue types like liver, extra-hepatic sites like PBMCs, serum and nervous tissue etc (Roque Afonso, Jiang et al. 1999). Laskus et al. studied PBMCs and autopsies from a broad range of HCV positive immunocompetent subjects and found variations in the 5'UTR within the quasispecies found in PBMCs which were not found in other tissues. Moreover, results also suggested the ability of PBMCs to adsorb quasispecies with different E2 related nucleotide sequences compared to the liver and serum (Laskus, Radkowski et al. 2000). As PBMCs have limited life span, the formation of unique quasispecies variants could be explained by replication of viral RNA within these cells (Roque-Afonso, Ducoulombier et al. 2005).

1.10. Peripheral blood mononuclear cells as reservoir for HCV

Hepatitis C is usually classified as a hepatotropic virus but many studies show that it may also undergo productive replication in other cells types. Numerous publications suggest that HCV can persist in peripheral blood mononuclear cells (PBMCs) as quiescent virus. However, whether persistence of HCV RNA and viral proteins indicate significant viral replication remains controversial. A number of studies have reported that cells belonging to the T and B cell lineage and monocytes/macrophages can

sustain HCV RNA even after treatment and this persistence of HCV replication in PBMCs could have a role in the late recurrence of HCV even after apparently successful treatment and may indicate a possible role of monocytes/macrophages acting as sanctuary sites for HCV (Sansonne, Iacobelli et al. 1996, Laskus, Radkowski et al. 2000, Bain, Fatmi et al. 2001). In one study HCV RNA was detected in monocytes and B cells of HCV patients (HBsAg negative and HIV seronegative patients). Moreover, as discussed above, PBMCs containing unique variants with nucleotide sequence that differed from HCV in other sites have been identified, suggesting that either viral replication and persistence may take place in these cells or that the cells differentially take up different HCV quasispecies (Ducoulombier, Roque-Afonso et al. 2004). Additionally, many other RNA and DNA viruses are also lymphotropic (Oldstone 1996) and these immune cells clearly demonstrate abnormal functions when infected with hepatitis C infection (Ducoulombier, Roque-Afonso et al. 2004).

Replication is evident in PBMCs when negative (anti-genomic, minus, anti-sense) stranded HCV RNA intermediates are generated but there is a lot of controversy about the assays used to detect negative strand RNA. The demonstration of HCV negative strand RNA is regarded as the hall mark of viral replication since viral proteins and HCV positive strand RNA may be present in the cells as a consequence of engulfment rather than replication. Moreover, it has been suggested in a few studies that the presence of HCV RNA in mononuclear cells could be from viral genomes trapped inside cells or adsorbed onto the cell surface (Lanford, Chavez et al. 1995, Laskus, Radkowski et al. 1997, Meier, Mihm et al. 2001). In previous studies

detection of negative strand has been performed by reverse transcriptase polymerase chain reaction which detects HCV RNA (Kao, Chen et al. 1997) and which uses a positive strand primer to specifically bind to negative strand RNA and initiate the amplification of negative strand. It is well known that the specificity of the product is critically dependent upon the specificity of the initiating primer. Various studies have debated that strand specificity could be an issue and considered the possibility that positive strand RNA could act as a template and lead to false priming of 'wrong' strands (Gunji, Kato et al. 1994, Lanford, Sureau et al. 1994). Furthermore, it was speculated that non-strand specific self-priming, because of strong secondary structures or contamination of nucleic acid, could lead to random priming and non-specific structures formation (Gunji, Kato et al. 1994, Lanford, Sureau et al. 1994). Various measures have been taken to eliminate these issues which include blocking 3 prime end of RNA chemically which allows only the relevant primer to be elongated by RNA polymerase (Gunji, Kato et al. 1994). This was undertaken by using tagged RT PCR in which a non-HCV tag is attached to the cDNA during its synthesis leading to the amplification of only the tagged cDNA by using a primer specific to the tag attached concurrently with the HCV oligonucleotide containing primer (Lanford, Sureau et al. 1994). Moreover, steps can be taken to limit contamination by doing both reverse transcription step and amplification step of PCR in the same tube. These steps have reduced but probably not eliminated the possibility of false positive results. However, detection of low level HCV replication remains problematic with no consensus on whether or not minuscule amounts of negative strand HCV RNA can be reliably detected or not.

Recent advancements to enhance the sensitivity of strand specific detection of anti-genomic HCV RNA by RT PCR have successfully demonstrated the presence of negative strand of HCV in PBMCs (reviewed in Sidorkiewicz, Grek et al. 2013). Carreño *et al*, showed that 5 out of 12 HCV RNA serum negative and anti-HCV positive patients had replicating HCV RNA in PBMCs, detected by strand specific real time RT PCR. However, it was observed that the occurrence of genomic positive strand was higher than the negative strand in PBMCs of all 5 patients. (Carreno, Pardo et al. 2006). MacParland et al. assessed infectivity of low level replicating HCV by exposing naive lymphocytes to the PBMCs from 9 subjects who had apparently achieved SVR. Negative strand was demonstrated in 4 out of 11 cultures through HCV RNA negative strand specific nested RT PCR combined with southern blot hybridization analysis of the subsequent amplicons while positive strand was seen in 11 out of 12 cultures. NS5A protein was seen by confocal immunofluorescence microscopy in the *de novo* infected cells. Additionally, it was found that the HCV variants found in T lymphocytes were different from those found in plasma indicating HCV replication or selective uptake of different quasispecies (see earlier) in them (MacParland, Pham et al. 2009). Most recently Keyvani et al. found occult HCV infection in 8.9% of the subjects by nested RT PCR from a cohort of 45 Iranian patients with cryptogenic cirrhosis selected for liver transplantation (Keyvani, Bokharaei-Salim et al. 2013).

Hypocholesterolemia is linked with chronic hepatitis C infection and HCV particle circulates in blood attached to lipoprotein. It has been shown that cholesterol and lipoproteins are essential for HCV uptake and replication

(Alvisi, Madan et al. 2011, Felmlee, Hafirassou et al. 2013). Sidorkiewicz *et al*, recently identified that intracellular cholesterol in PBMCs was low in the case of chronic hepatitis C patients who had anti-sense HCV RNA in their PBMCs. This suggests that hypocholesterolemia could be a harbinger of HCV persistence after SVR (Sidorkiewicz, Grek et al. 2013).

In one recent study, 55 patients undergoing treatment with PEG-IFN and ribavirin were studied for the relationship between anti-genomic HCV RNA in PBMCs (detected through strand specific RT PCR) and SVR/relapse. Out of 15 subjects who relapsed, 2 had negative strand before treatment and the proportion remained the same at the treatment end point which is in conformity with previously reported studies (Radkowski, Gallegos-Orozco et al. 2005, Mohamad, El-Bab et al. 2011). This suggests that HCV persists in PBMCs of the patients who relapse (Inglot, Pawlowski et al. 2013). Surprisingly, it was found that the rate of SVR was higher in individuals who had negative strands in PBMCs before treatment (10 out of 33 patients who achieved SVR) while none of the patients who achieved SVR had negative strand HCV in PBMCs after treatment (0 out of 33 patients). The exact reason, why more patients with replicating HCV in PBMCs before treatment showed SVR is a paradox. One of the suggestions is that the PBMC linked HCV variants might attenuate the immune response. Suppression of HCV replication in PBMCs by antiviral therapy might antagonise this negative effect on the immune system (Inglot, Pawlowski et al. 2013).

As discussed in the previous section, HCV exists in its host in the form of mutated quasispecies which help it to adapt to the host immune responses

by producing immune escape mutants. These quasispecies are found to differ from each other in their genomic sequences. It is mostly the hyper variable region (HVR1) which is more prone to mutations, perhaps in response to subjective pressures from humoral and cellular immunity (Bukh, Miller et al. 1995). It has been shown that the quasispecies present in PBMCs are different from those present in the serum and the liver of the same patient (Okuda, Hino et al. 1999). This has been used as supporting evidence for the notion that HCV replicates in PBMCs as the formation of quasispecies can only occur in presence of replication (Laskus, Radkowski et al. 1998, Lerat, Rumin et al. 1998, Navas, Martin et al. 1998, Laskus, Radkowski et al. 2000, Roque-Afonso, Ducoulombier et al. 2005, Di Liberto, Roque-Afonso et al. 2006). However, it is possible that PBMCs are infected by an entry mode that differs from that seen in hepatocytes and therefore it is plausible that differential uptake into PBMCs may be responsible for the varying quasispecies distribution rather than replication within monocytes per se. Nevertheless, the hypothesis that HCV does replicate in PBMCs has been supported by the data showing that HCV can be detected in PBMCs through *in situ* hybridization techniques in which only negative strands are detected (Sansanno, Iacobelli et al. 1996). Again the specificity of the technique is not beyond question and it remains controversial as to whether HCV does or does not replicate in non-hepatic reservoirs. Despite these reservations the extrahepatic persistence of HCV RNA has been put forward as a possible cause of the relapse of HCV after treatment.

Revie *et al*, looked at 75 RT PCR studies done to evaluate the presence of replicating HCV RNA in the liver and other human cells. 85% of the studies

tested positive in either one or more samples for negative strand intermediate in cells other than hepatocytes. The review also evaluated studies which looked at the viral replication through other methods like immunofluorescence, immunohistochemistry, *in situ* hybridisation and quasispecies analysis. These other methods also demonstrated that HCV RNA replicates in the extrahepatic cells (reviewed in Revie and Salahuddin 2011). The evidence that HCV causes a number of lymphoproliferative diseases (mixed cryoglobulinemia, non-hodgkin's lymphoma etc) apart the from liver infection, indicates that HCV also interacts with cells other than hepatocytes (Ferri, Greco et al. 1991, Pozzato, Mazzaro et al. 1994, Zignego, Ferri et al. 1997, Giannini, Petrarca et al. 2008). Apart from the negative strand, presence of NS proteins also signifies replication as they are not found in the viral particles and are formed during the replication process.

In order to replicate HCV should first bind and then enter the cell. In one study the binding of soluble form of HCV E2 (sE2) protein with different types of PBMCs was analysed. It was found that sE2 binding was parallel to expression of CD81. However, monocytes, B cells and dendritic cells had some independent factors which supported binding (Yamada, Montoya et al. 2005). Another study that looked at the binding and internalising of antibody bound core and E1 into PBMCs found that in one hour these proteins were found on the surface of the cells while after 24 hours surface staining was absent and 28% cells showed intracellular staining. This was further confirmed by the presence of negative strand on RT PCR (el-Awady, Tabll et al. 2005). Marino *et al*, showed that stimulating monocyte (U937 and monomac-6) and T lymphocyte (MOLT-4 and jurkat) cell lines by IFN- γ and

phorbol myristate (PMA) to up-regulate Fc receptor expression enabled the immune complex bound HCV entry into the monocytic but not the lymphocytic cell lines. Furthermore, anti-genomic strands were detected for 7 days in permissive cell lines (Marino, Deibis et al. 2005).

As discussed above, HCV replication in host cells entails conversion of viral genome into anti-genomic strand and then back into positive strand. Therefore, negative strand within the cell signifies replication process. Initially many investigators showed that PBMCs contain negative strands by using regular PCR (Bouffard, Hayashi et al. 1992, Henin, Makris et al. 1994, Manzin, Candela et al. 1994, Muller, Kallinowski et al. 1994, Willems, Peerlinck et al. 1994, Lohr, Goergen et al. 1995, Ounanian, Gueddah et al. 1995, Crovatto, Pozzato et al. 2000, Karavattathayil, Kalkeri et al. 2000, Goutagny, Fatmi et al. 2003, Natarajan, Kottilil et al. 2010). Bone marrow cells, brain and oral tissues were also found to contain negative strands (Fong, Shindo et al. 1991, Horiike, Nonaka et al. 1993). PCR is a sensitive technique in which viral RNA is converted into DNA by reverse transcriptase (RT). Previously nested PCR was used to acquire amplicons from a specific region of genome. The main issues with regular PCR are the formation of false positives due to self-priming or priming with contaminated RNA or DNA in the reaction mix. Steps to improve the efficiency and accuracy of PCR were tested, e.g.; inactivation of reverse transcriptase (RT) by either proteinase K or extended heating, RNase mediated removal of RNA and dilution of RT. These steps reduced the chances of false positives considerably. Some studies used tagged primers (additional sequence at 5' end which was not related to the amplicon) for RT step and rTth polymerase

(functions at high temperature i.e.; 70-74°C, to reduce chances of false positives). The need to perform nested PCRs is also reduced due to availability of systems which can detect very low level of RNA (Sangar and Carroll 1998).

Revie *et al*, evaluated studies which used PCRs with either one or more modifications discussed above. Almost all studies reported the existence of replicating intermediate (minus-sense RNA) in PBMCs (Takehara, Hayashi *et al.* 1992, Wang, Sheu *et al.* 1992, Gunji, Kato *et al.* 1994, Navas, Castillo *et al.* 1994, Shindo, Di Bisceglie *et al.* 1994, Yun, Sonnerborg *et al.* 1994, Artini, Nisini *et al.* 1995, Lanford, Chavez *et al.* 1995, Muratori, Giostra *et al.* 1995, Taliani, Badolato *et al.* 1995, Chang, Young *et al.* 1996, Lerat, Berby *et al.* 1996, Mihm, Hartmann *et al.* 1996, Kao, Chen *et al.* 1997, Laskus, Radkowski *et al.* 1998, Lerat, Rumin *et al.* 1998, Mellor, Haydon *et al.* 1998, Laskus, Radkowski *et al.* 2000, Lin, Fevery *et al.* 2002, Authier, Bassez *et al.* 2003, Gong, Lai *et al.* 2003, Fournillier, Freida *et al.* 2004, Nielsen, Bassendine *et al.* 2004, Pham, MacParland *et al.* 2004, Blackard, Smeaton *et al.* 2005, Carreno, Pardo *et al.* 2006, Sansonno, Tucci *et al.* 2007, Pham, King *et al.* 2008, Inokuchi, Ito *et al.* 2009, Lin, Libbrecht *et al.* 2009). However, despite numerous studies suggesting the presence of replicating virus in PBMCs, this subject remains controversial. The fact that the ratio of positive to negative strand in extrahepatic cells is 10 to 1 respectively and the negative strands in extrahepatic sites are one tenth of the amount of negative strands in hepatocytes, proves that the ratio of negative strand in hepatocytes and PBMCs will be 1 to 1000 respectively (Fong, Shindo *et al.* 1991, Laskus, Radkowski *et al.* 1998). Moreover, the studies which found

replicating RNA in the extrahepatic cells did not consistently find it in all patient samples, the reason for this is not clear.

In one series, mothers containing anti-sense viral RNA in their PBMCs transmitted HCV infection to their babies compared to those without replicating anti-sense viral genome in their PBMCs (Azzari, Resti et al. 2000). However, the confounding factor could be difference in concentration or some indeterminate factor as not all mothers who transmitted infection had negative strand in their PBMCs. In another series, it was found that HCV positive individuals who used intravenous drugs were more likely to have negative strands in their PBMCs compared to non-IVDU HCV positive subjects (Resti, Azzari et al. 2002). IVDU might lower the immune response to the virus but this could also be due to prolong time after infection. These studies suggest that negative strands are present in PBMCs at varying amounts and different factors affect the likelihood of them getting detected.

In situ hybridisation (ISH) technique was used to detect negative strand in PBMCs in various studies. It is three times less sensitive than RT PCR. In addition negative strand concentration is much less than the positive strand (Agnello, Abel et al. 1998). Moreover, detection of single stranded RNA could be impaired due to the formation of double strand by negative and positive strands. This problem is however tackled by denaturing tissue RNA at 95°C before probe is added but there is a potential of underestimating the presence of infection in cells by this technique. Many studies done to detect viral replication by ISH have provided evidence of the presence of replicating intermediates in PBMCs (Moldvay, Deny et al. 1994, Tian, Yang et al. 1998,

Rodriguez-Inigo, Casqueiro et al. 2000, Castillo, Rodriguez-Inigo et al. 2005). HCV positive strand was detected in 18 patients with occult HCV infection in one study by using RT PCR and fluorescent ISH. Negative strand was detected in 61% of those with concordant results between the two methods (Castillo, Rodriguez-Inigo et al. 2005).

Another way of analysing HCV replication is by the analysis of viral protein through immunostaining. Viral protein formation is an essential step in HCV replication as discussed previously. The disadvantages of using this approach are that low level HCV might make viral protein detection problematic. Also antibodies against NS proteins might not react with NS proteins in every sample which makes it essential to test various antibodies before choosing the one which gives accurate results (reviewed in Revie and Salahuddin 2011). The majority of immunohistochemistry and immunofluorescence studies have demonstrated the presence of viral proteins in PBMCs. Various antibodies against NS3, NS4, NS5 and core protein have been used and detected HCV proteins in PBMCs (Blight, Lesniewski et al. 1994, Sansonno, Cornacchiulo et al. 1995, Gong, Lai et al. 2003, Benkoel, Biagini et al. 2004, Pal, Sullivan et al. 2006, Pham, King et al. 2008, Coquillard and Patterson 2009, Sir and Kim 2010).

Quasispecies analysis of HCV in the extrahepatic cells has been performed by the single-strand conformational polymorphism analysis (SSCP) and by the cloned sequence variants. 5'UTR and HVR1 have been looked at most frequently in many studies. In SSCP bands from different tissues are sequenced, however one band could contain more than one variant leading

to underestimation of the results. Various studies have compared liver variants with the other tissues and found differences between variants present in different sites (Maggi, Fornai et al. 1997, Navas, Martin et al. 1998, Radkowski, Wang et al. 1998, Okuda, Hino et al. 1999, Roque Afonso, Jiang et al. 1999, Bain, Fatmi et al. 2001, Laskus, Radkowski et al. 2002, Radkowski, Gallegos-Orozco et al. 2005, Pal, Sullivan et al. 2006, Blackard, Hiasa et al. 2007, Ramirez, Perez-Del-Pulgar et al. 2009, Bokharaei Salim, Keyvani et al. 2010, Li, Sullivan et al. 2010, Ramirez, Perez-del-Pulgar et al. 2010). Revie *et al*, examined 44 studies, out of which 43 studies showed large differences in the quasispecies variants among different tissue types while only one study showed just a small difference among variants (reviewed in Revie and Salahuddin 2011). Many studies found quasispecies difference in HVR1 and 5'UTR among the liver, serum and extrahepatic cells (Maggi, Fornai et al. 1997, Forton, Karayiannis et al. 2004, Vera-Otarola, Barria et al. 2009, Li, Sullivan et al. 2010). It was also found that some variants had higher replicative capacity compared to the others and some tissues like brain resided variants showing lower translation frequency (Forton, Karayiannis et al. 2004). These studies suggest that the quasispecies difference affects the replicative capacity of HCV in different tissues. It was observed that in some patients there was considerably high quasispecies variability between two type of cells and this variability increased with time, hence longer the time of infection, higher the quasispecies variability amongst different tissues (Cabot, Esteban et al. 1997, Cabot, Martell et al. 2000, Cabot, Martell et al. 2001). In a study following 4 patients for quasispecies diversity during a course of 18 years, it

was observed that HCV evolved through four stages in which it established an infection, variants evolved into subpopulations, diversification of variant subpopulations took place and new subpopulations emerged and in the last stage negative selection took place leading to adaptation to the host environment. However, the major drawback of this study was a small sample size (Ramachandran, Campo et al. 2011). This variation in quasispecies gene pool could either be due to the ability of HCV to adapt to different environments in different tissues or due to different binding capacities of different tissues selecting some variants and rejecting the other.

By infecting significant proportion of PBMCs, such as B and T lymphocytes, monocytes, macrophages and dendritic cells, HCV could interfere with both the innate and adaptive immune responses of an individual (Blackard, Smeaton et al. 2005, Blackard, Hiasa et al. 2007). By infecting immune cells and interfering with their ability to mount an immune response HCV persists in the host. The pathogenesis of extrahepatic disease could be explained by the ability of HCV to infect lymphocytes. Furthermore, if PBMCs are reservoirs of HCV RNA, they could contribute to re-infection of a graft after transplantation. Reports that PBMCs could be detected in the individuals whose serum HCV RNA disappeared after treatment could be the reason why the viral relapse took place in these patients (Marukian, Jones et al. 2008).

1.10.1. Role of monocytes/macrophages in harbouring HCV

Replication of HCV in extrahepatic sites has been under research for more than a decade and as discussed in previous section, one of the

compartments thought to house HCV is the PBMCs. However, due to the low level viral replication this remains a controversial issue. Monocytes and macrophages are known to house many replicating viruses. They are permissive to a wide range of viruses including other flaviviruses (Mogensen 1979). Monocytes/Macrophages are thought to harbour sanctuary sites for HCV viral persistence even after treatment with standard HCV antiviral therapy and may act as Trojan horses for 'occult' HCV infection. They might have a role in the relapse after treatment and re-infection of the graft after transplantation.

'Occult' HCV infection is described as the presence of HCV RNA in the liver tissue and PBMCs (detected in 70% of the cases with occult infection), after clearance of HCV RNA and anti-HCV antibodies from the serum of an individual in response to anti-HCV therapy. Occult infection can be identified in 87% cases after combining the results of HCV RNA detected in PBMCs and ultracentrifuge serum samples. This could be a step towards detecting occult infection without the need for liver biopsy (Bartolome, Lopez-Alcorocho et al. 2007). Infection in PBMCs has also been reported to have a link with the presence of anti-HCV antibodies and normal ALT levels years after resolution of infection (Pham, MacParland et al. 2004, Radkowski, Gallegos-Orozco et al. 2005, Radkowski, Horban et al. 2005).

Replicating forms of HCV RNA have also been reported in the monocytes/macrophages from HCV and HIV co-infected individuals (Laskus, Radkowski et al. 2000). Naïve human monocytes/macrophages are reported to be susceptible to infection with HCV in tissue culture and it has been

shown that the concomitant HIV infection facilitates the HCV infection *in vitro* (Laskus, Radkowski et al. 2004). Numerous studies have shown evidence of active HCV RNA replication in HIV and HCV co-infected patients (Laskus, Operskalski et al. 2007, Laskus, Radkowski et al. 2004, Laskus, Radkowski et al. 1998, Laskus, Radkowski et al. 2000). Human immunodeficiency virus has been shown to facilitate HCV replication in monocytes/macrophages of the host and this may help to explain why mothers co-infected with HIV and HCV are more prone to transmit HCV infection to their off springs (Granovsky, Minkoff et al. 1998, Thomas, Newell et al. 1998). Furthermore, it is thought that the viral dormancy in PBMCs, even after treatment induced clearance of HCV from serum, can lead to the reactivation of infection especially under conditions of immune suppression and this might have a role in the extrahepatic manifestations of HCV infection (Ferri, Monti et al. 1993). Various studies have confirmed the relationship between the viral relapse and the presence of replicative intermediate in the extrahepatic sites especially PBMCs (Xu, Xie et al. 2005, Majda-Stanislawski, Bednarek et al. 2006, de Felipe, Leal et al. 2009). However, testing extrahepatic cells for HCV replication is not done routinely in order to assess the treatment outcome. Moreover, it is not known whether HCV negative strands in PBMCs effects the treatment response.

HCV infection in monocytes was first reported in the monocytes/macrophages of HIV and HCV co-infected patients (Laskus, Radkowski et al. 2000). Laskus et al. found that two out of seven cultures of purified monocytes/macrophages had negative strands isolated for 21 days. Radkowski et al. isolated fresh macrophages from healthy subjects and

subjected them to the sera of HCV positive patients. 16 out of 25 cultures produced HCV negative strands for 14-21 days. SSCP and sequence analysis of the 5'UTR region showed that 4 cultures had different sequence variants compared to the initial sera. Moreover, the infected cultures had larger quantities of IL-8 and TNF- α compared to the uninfected cultures (Radkowski, Bednarska et al. 2004). The same group then looked at the infection of healthy monocyte/macrophages first with HIV and later with HCV. It was found that all cultures which were infected with HIV showed HCV replication whereas HCV infected cultures showed infection only in 50%, concluding that HIV might facilitate HCV infection in macrophages (Laskus, Radkowski et al. 2004). Similarly, studies with immature- and mature-dendritic cells showed positive strands for 10 and 5 days respectively while negative strand was seen for 1-2 days in both types (Navas, Fuchs et al. 2002).

The JFH-1 genome from a patient with fulminant hepatitis contains a virulent strain of HCV and is able to produce infection in Huh 7.5 cells; however studies have failed to demonstrate the infection of *in vitro* monocytes, macrophages, DC cells, B-cells and T-cells through JFH-1 (Marukian, Jones et al. 2008). Though able to infect a permissive cell line (Huh 7.5 cells), this could be one of the setbacks of the *in vitro* derived HCV which may not mimic the HCV infection of PBMCs *in vivo*. Also Huh 7.5 cells are hepatoma derived cells with defective HFE protein (human haemochromatosis protein) (Vecchi, Montosi et al. 2010) and RIG-I (RNA helicases) (Sumpter, Loo et al. 2005). Therefore, synthetic models should be approached with caution as they

might not truly replicate *in vivo* results. Furthermore, the unusual genome (JFH-1) could behave differently to typical HCV infections.

Caussin-Schwemling et al. infected monocytes/macrophages obtained from healthy donors by subjecting them to sera of genotype 1b positive HCV patients. Low level viral replication was seen on RT PCR. Sequence analysis of HVR1 showed distinct variant tropism to monocytes/macrophages (Caussin-Schwemling, Schmitt et al. 2001).

Cultures containing HCV infection within PBMCs have been produced by many investigators, however HCV levels start dropping after 3 weeks which is most likely due to the maturation of macrophages after 2 weeks of culturing, thus releasing cytokines that inhibit other cell types (Salahuddin, Markham et al. 1982). Revie et al. developed a culture system by infecting macrophages obtained from umbilical cord blood with the plasma or serum of HCV infected individuals and its supernatant was used to infect other cell types such as B and T cells, lymphoid cells and neuronal cells (Revie, Braich et al. 2005). It has been speculated that macrophages might modify the HCV virus by up-regulating certain factors which promote its infection or select variants which could be replicated in PBMCs. As infection of Huh 7.5s has not been carried out through this system, it is not known whether the HCV variants selected through macrophages would infect hepatocytes. In another series from the same group it was reported that the cord blood macrophages were not susceptible to infection by genotype 3 strain of HCV and it was presumed that these macrophages might preferentially select genotype 1 HCV (Revie, Alberti et al. 2010). It is not clear how HCV infects monocytes

and macrophages and whether HCV uses the Fc receptor to internalise into macrophages or not. Few *in vitro* studies with macrophages suggest that genotype 1 HCV might preferentially proliferate in macrophages (Revie and Salahuddin 2011) and it is suggested that this may explain the resistance of this genotype to interferon based therapies.

Coquillard et al. used simultaneous ultra-sensitive subpopulation staining/hybridization *in situ* (SUSHI) to assess HCV infection in different subsets of monocytes acquired from HCV infected individuals, with and without HIV co-infection. It was determined that CD14⁺⁺/CD16⁺⁺ and CD14⁺/CD16⁺⁺ monocytes showed HCV infection while CD14⁺⁺/CD16⁻ monocytes did not. This selective monocyte tropism was analysed further and it was found that permissive subsets had up-regulation of CD81 receptors on their surfaces. Nonetheless, this study only looked at infection from genotype 1 strain of HCV and the findings cannot be reciprocated with other genotypes (Coquillard and Patterson 2009).

In summary, the work to-date on HCV entry and replication in PBMCs is inconclusive. There is a potential for artefacts in the studies of PBMCs' compartmentalisation due to sampling error induced by low copy numbers and cloning or SSCP approaches. However the overall weight of the evidence is for some entry of HCV into PBMCs and, on balance, it seems probable that low level viral replication does take place within some of the cells. Replication may be enhanced by the presence of HIV.

1.11. Fusion

Fusion is an important cellular process in which cells with single nucleus come close together and make multinuclear cell through a complex process preceded by membrane fusion. Although the process of viral fusion to cells is well understood, the exact mechanism by which cell-to-cell fusion takes place is still largely unknown. It involves different pathways and co-factors which orchestrate a process of cell fusion. This mechanism is seen in different physiological processes in the body such as the differentiation of bone, muscles and trophoblast cells, during embryogenesis, and during morphogenesis. It is well recognised that biological fusion of cellular membranes has a role in cell and molecular trafficking, endocytosis/exocytosis and viral entry. Cell membranes have intracellular pockets which undergo fusion and fission to perform these roles (Chen, Grote et al. 2007). Cell-to-cell fusion is a specialised type of membrane fusion. Recent years have put more emphasis on understanding of the diverse role of cell-to-cell fusion in the field of development, regeneration and homeostatic regulation of an organism. Viruses contain membrane fusion glycoproteins which enable their fusion with host cell membrane and mediate transmission of viral particles into the host cells. These attachment proteins create initial contact between the viruses and cells and mixing of two membrane bilayers occur (Skehel and Wiley 2000, Kielian 2006). It is well known that both monocytes derived dendritic cells and those residing in the tissues bear receptors for virus envelope glycoproteins. They may enable dissemination of viruses to lymphoid cells through these receptors. One such example is dendritic cell mediated transmission of HIV to T lymphocytes through the cell surface

receptors (i.e. DC-SIGN) (Zoetewij and Blauvelt 1998, Engering, Van Vliet et al. 2002, Geijtenbeek and van Kooyk 2003).

1.11.1. Mechanism of cellular fusion

There are various molecular hypotheses about how cell-to-cell fusion takes place but the exact mechanism remains an enigma. It is said to be an important cellular event which plays a major role in many physiological and pathological processes. Molecular events which orchestrate the fusion process are thought to be cell type and species specific but are known to share some similarities (Chen, Grote et al. 2007). One hypothesis is that cells enter a pre-fusion stage prior to fusion under the action of pre-fusion signals and cell membranes undergo modulation and orient in a specific fashion to mediate adhesive interactions between two cells via specialised transmembrane proteins. These interactions can either stabilise the membranes for fusion or directly initiate the fusion process (Chen, Grote et al. 2007).

1.11.2. Biological role of cell-to-cell fusion

Multicellular organisms exhibit cellular fusion in a broad range of physiological and pathological processes. Some cells are thought to be fusion-competent and exhibit properties which trigger the process while others acquire these characteristics secondary to specialised signals (Helming and Gordon 2009). Fertilization of sperm and ovum resulting in formation of zygote demonstrates fusion in which two haploid genomes come together in one single hybrid which has properties different to its parent cells.

Myoblast fusion contributes to excessive muscle mass by increasing the myofibre size and diameter and hence therapy based upon myoblast fusion may be used to treat muscular dystrophies (Skehel and Wiley 2000, Chen, Grote et al. 2007). Similarly fusion of monocyte/macrophages in the development of osteoclast and fusion of trophoblasts in the placenta are good examples of the physiological roles of fusion. Genetic reprogramming of somatic cells can take place after fusion with stem cells and may contribute to tissue regeneration. Transplanted cells incorporate in regenerating organs by fusion and contribute to tissue mass (Chen, Grote et al. 2007, Lluís and Cosma 2010). Pathologically, during inflammation fusion of macrophages can lead to multinucleated giant cell formation and aberrant fusion process can contribute to cancer formation and progression (Chen, Grote et al. 2007, Duelli and Lazebnik 2007, Oren-Suissa and Podbilewicz 2007, Podbilewicz 2006). Additionally, disruption of normal fusion process can lead to osteopetrosis (Chen, Grote et al. 2007). Cellular fusion has also been implicated in the metastasis of cancer cells. The initial theory dates back to 1871 when it was proposed that fusion between immortalized cancer cells and leukocytes potentiate the hybrid cells to settle in distant areas in the body (Pawelek and Chakraborty 2008). This information has driven research in this area in order to derive therapeutics to counteract fusion process to prevent tumour progression.

1.11.3. Characteristics of fused (hybrid) cells

New hybrids are formed after cell-to-cell fusion and are thought to have different characteristics compared to their mono-nuclear predecessors. This

is thought to be due to reprogramming of cells. Studies have shown that if a somatic cell is fused with a stem cell a hybrid with characteristics specific to stem-cell might emerge due to reprogramming (Lluis and Cosma 2010, Duelli and Lazebnik 2007, Oren-Suissa and Podbilewicz 2007, Podbilewicz 2006, Sapir, Avinoam et al. 2008). When cells from two different lineages fuse they result in the formation of a *heterokaryons* or *synkaryon*. Heterokaryon is a hybrid cell which is of heterologous nature because it contains two nuclei which have not fused. Synkaryon is a fused cell in which nuclei fuse (*karyogamy*) and contain double the normal genetic material i.e.; hybridoma cells formed after fusion of murine myeloma cells with B cells of an immunised mouse (Lluis and Cosma 2010).

1.12. Rationale for the studies in this thesis

There is currently no model system whereby HCV replication from patients infected with diverse genotypes can be readily studied. The heterogeneity of HCV and the lack of a readily available HCV replication culture containing patients' isolates have contributed to the failure to develop effective direct acting antiviral drugs against HCV genotypes other than genotype 1. Furthermore, the development of vaccine against HCV and the study of neutralizing antibodies have also been hampered. With therapeutic advancements in the development of novel antivirals for HCV it will be increasingly important to examine the replication of HCV from individual patients to assess the drug sensitivity and to determine which combination of antiviral agents is most suitable. We speculate that a novel replication system for HCV will have widespread clinical and academic uses and the goal of this

thesis is to examine different approaches to develop such a system. We have adopted two different approaches to develop a new model for HCV infection: one involves an *ex vivo* liver biopsy model using liver tissue from patients with chronic HCV infection and the second involves a cell fusion model in which monocytes from patients with hepatitis C, which are known to harbour virus, are fused with a liver cell line (Huh 7.5s) to generate hybrid, putatively infected cells. Here I will describe the experiments and results of these studies with emphasis on their technical creation, extent of HCV viraemia, infectivity rate and validation by the evaluation of antiviral strategies.

1.13. Aims and background

As discussed earlier hepatitis C infection is a global health problem with an estimated 130-170 million (3% of global population) infected worldwide. Out of the six main genotypes of HCV, viral genotypes 1, 2 and 3 are most prevalent in the UK. Therapy with PEG-IFN and ribavirin cures no more than 50% of patients infected with genotype 1 HCV although response rates are increased with other genotypes. New antiviral drugs specifically targeted to HCV have the potential to be effective in greater proportions of individuals and result in fewer adverse effects and the first wave of direct acting antiviral agents are currently widely used for patients with genotype 1 infection.

The prospects of preclinical drug testing is hampered by the lack of a cell culture system which harbours naturally occurring hepatitis C virus derived from the hepatitis C patients. To optimally assess the efficacy of new drugs and to identify the drug resistance we need HCV cultures for different viral strains derived purely from infected individuals. Hepatitis C virus shows poor

replication *in vitro* and currently novel ant-viral drug testing entails reliance on the viral replicons which are derived from single viral isolates. Recent advances have allowed HCV clones to be propagated in the human hepatoma cell line but these viral derivatives (replicons) depend on tissue culture adaptive mutations and subsequently lead to the alteration of function. Some culture systems are limited to the non-structural gene of a single HCV genotype 2a genome (unique strain - JFH-1) derived from a single patient. A robust infectious system with clinical isolates of heterogeneous nature representing naturally occurring virus does not exist.

The aim of the work described in this thesis is to develop models which allow diverse genotypes and patient derived virions to be cultured thus enabling novel drug testing to discover the effect of new drug therapies, individual genotype drug response and sensitivities. This was carried out by developing two models, an *ex vivo* liver biopsy model and an *in vitro* HCV fusion replication model.

Hypotheses to be tested:-

1. Fragments of liver tissue from patients with chronic HCV can be cultured *in vitro* and will contain replicating HCV that will be sustained for sufficient time to assess the replication of HCV.

To examine this a short term *ex vivo* culture system of liver fragments, isolated from the biopsies performed on HCV patients, was developed. The liver biopsy culture system was tested for hepatocyte viability in tissue culture and for the response to different antiviral drugs on heterogeneous genomes

of clinically relevant genotypes. As primary human hepatocytes do not survive for long in tissue culture we looked at a variety of ways to maintain hepatocytes phenotype and to prolong their survival. Furthermore, we assessed replication of HCV and monitored its inhibition with a variety of antiviral agents, specifically telaprevir and the experimental compound NA808.

2. Replication of HCV in monocytes can be amplified by fusion with hepatocyte cell lines.

As discussed above monocytes of HCV patients are said to harbour HCV, it is however not clear whether HCV replicates in these cells or is adsorbed on the cell surface. Work in this thesis was based on speculation that fusion of monocytes from HCV patients belonging to diverse genotypes with immortalised liver cells (Huh 7.5s) would enhance or maintain HCV replication.

Chapter Two

Materials and Methods

2.1. Materials

2.1.1. Liver biopsy culture system

A) Liver biopsy samples

Samples of infected liver tissue were obtained from surplus tissue excised from routine liver biopsy samples or from non-malignant tissue from liver cancer resection margins at the *Royal London Hospital*. Approval for this study was obtained from *The East London and City Research Ethics Committee* and informed consent was obtained in accordance with the Declaration of Helsinki. Samples were processed immediately within an hour. All experiment with samples obtained from hepatitis C patients were carried out in the containment level 3 laboratory.

B) Cell culture consumables

All tissue culture consumables (tissue culture flasks, pipettes, micropipette tips, culture plates) were purchased from VWR International Ltd UK, unless stated otherwise.

C) Cell culture reagents

Dulbecco's modified Eagle Medium (DMEM), foetal calf serum (FCS), non-essential amino acids, L-glutamine, penicillin-streptomycin, trypsin, Dulbecco's phosphate buffer saline (PBS), and all other chemicals were supplied by Sigma-Aldrich Ltd-UK, unless otherwise stated. CellTiter-Glo®

Luminescent Cell Viability Assay from Promega Ltd-UK was used to assess viability of liver fragments.

D) Drugs used in experiments

Drugs	Suppliers	Concentration
Interferon-Alpha 2a	Roferon,Roche UK	500IU/ml
Telaprevir Vx950 (Protease Inhibitor)	Virgil European Consortium Drug Pharma team	14.7nM
NA808 (Serine	Chugai Pharma	50nM
Palmytoiltransferase Inhibitor)	Co. Ltd. Japan	500nM

Table 2.1: Drugs used on HCV infected liver biopsy samples; interferon-alpha, telaprevir, NA808 and their suppliers.

E) Reagents used in RNA extraction and cDNA formation

Products	Suppliers
TriZOL®	Invitrogen UK
Propanol for molecular biology minimum 99%	Sigma-Aldrich Ltd UK
Chloroform minimum 99%	Sigma-Aldrich Ltd UK
RQ 1(RNA Quantified)	Promega UK
RNase-free DNase	
RQ 110X reaction buffer (Tris-HCL, MgSO ₄ , CaCl ₂)	Promega UK
RQ 1 DNase Stop solution (EGTA)	Promega UK
Oligo (dt) 15 primer	
M-MLV	Promega UK
Reverse transcriptase	

M-MLV RT 5X Buffer	Promega UK
dNTP RNasin® RNase inhibitor	Promega UK

Table 2.2: Reagents used in both RNA extraction and formation of cDNA.

F) Reagents used in PCR reaction

Primers for HCV-NTR (p1-2, p3-4), B-actin, MxA and 2 5' OAS were purchased from Sigma-Aldrich Ltd UK and 2x Quantitect SYBR green mix was supplied by Qiagen UK.

G) Hepatocyte supplement medium

Hepatocyte supplement medium contains Dulbecco's modified eagle medium (DMEM) with high glucose, 10% foetal calf serum, L-glutamine, 0.5U/ml insulin, 7ng/ml glucagon, 7.5 µg/ml hydrocortisone (insulin, glucagon and hydrocortisone were purchased from Sigma Aldrich UK) and 1%penicillin-streptomycin.

H) Collagen type 1

Collagen type 1 was used to coat the bottom of wells used for growing the hepatocytes. It was purchased from (Invitrogen UK).

I) Fibroblast culture medium

Fibroblast culture medium contains Dulbecco's modified eagle medium (DMEM) with high glucose, 10% foetal calf serum, L-glutamine and 1% penicillin-streptomycin.

J) Primary human hepatocytes

Primary human hepatocytes (PHHs) were isolated from surplus liver biopsy tissues from HCV patients by the technique described below.

K) 3T3 murine embryonic fibroblasts

3T3 Murine embryonic fibroblasts were a kind gift from the Centre for Cutaneous Research, Blizzard Institute of Cell and Molecular Science. These fibroblasts were irradiated by transferring them into 15 ml falcon tubes in 10 ml DMEM (2×10^6 cells per ml) and gamma-irradiated at 5000 rads for 20-30 minutes. Fibroblasts were then incubated with hepatocyte supplement medium after being 60-70% confluent in DMEM (10% FCS). Hydrocortisone and insulin in hepatocyte supplement medium were meant to prevent cell division and avoid over-confluence of fibroblasts.

L) Isolation of primary human hepatocytes

A few different approaches to separating hepatocytes from the liver biopsy fragments were tried. Both mechanical and enzymatic (Collagenases) separation techniques were tested. Optimal mechanical dissociation

approach was adopted for all the biopsies. Enzymatic technique using collagenases did not give a higher yield of PHHs from liver biopsies compared to mechanical dissociation. Cells were gently scraped from the biopsy tissue using small cell scraper (18 mm blade) and pipetted up and down gently to dissociate them from fibrous tissue. Fibrous tissue was separated out using a cell strainer and cells were centrifuged at 500 rpm for 5mins at 4°C to separate them from medium. Hepatocytes were assessed for viability using trypan blue selection (75-90%).

M) Pure hepatocyte culture

For pure hepatocyte cultures, PHHs were seeded in wells of polystyrene omnitrays which were uniformly coated with rat tail collagen type 1 (From Invitrogen UK).

N) Co-culture technique

Type 1 collagen solution (90-100µg/ml) made in distilled water was incubated in wells of 12 and 24 well tissue-culture polystyrene omnitrays (Nunc from Sigma-Aldrich, UK) for 1 hour at 37°C. Any excess collagen was aspirated from these wells. Either irradiated or non-irradiated 3T3 fibroblasts were grown in these wells for 1 day to reach 40-50% confluence. They were incubated in fibroblast culture medium (see above). PHHs were seeded over fibroblasts after 1 day and fibroblast supplement medium was replaced by hepatocyte supplement medium (300 µl and 800 µl for 24 and 12 well plate respectively). Fibroblast over growth was prevented by the presence of

hydrocortisone and insulin present in the hepatocyte supplement medium – as these drugs reduce the proliferation of fibroblasts. Hepatocyte to fibroblast ratio was estimated to be 4:1. Haemocytometer was used to count cells.

2.1.2. Materials used for hepatocyte fusion model

2.1.2.1. Cell lines

A) Monocytes from hepatitis C patients

40-50mls of Blood was obtained from treatment naïve patients with active hepatitis C at *Royal London Hospital*. Ethical approval for the study was obtained from *Moorefield and Whittington Research Ethics Committee in London*. Patients were consented in accordance to the declaration of Helsinki. Monocytes were extracted from the patients' blood as described in section 2.2.2.2 and 5-10 million monocytes were used in each experiment. All samples obtained from hepatitis C patients were handled in the containment level 3 laboratory.

B) Huh 7.5 cell line (human hepatoma cell line)

Huh 7.5 cell line was obtained from Dr Michael McGarvey, from Imperial College London and was originally developed by Dr Keril Blight who obtained this cell line from a subpopulation of Huh 7 cell line after eliminating replicon from the most permissive Huh 7 cells. Replicon-containing Huh 7 cells were first obtained by growing them in non-selective medium for a week and subsequently treating with IFN- α for 3 to 4 days to eliminate replicon. This

led to formation of Huh 7.5 cells which is the most viral replication permissive 'cured' sub-line (Blight, McKeating et al. 2002). Before fusion 1×10^6 Huh 7.5 were grown in 75 mm³ flasks until 70-80% confluent so that they were in the exponential growth phase before fusion.

C) Replicon cell line

Replicon cell line was a kind gift from Dr Michael McGarvey from Imperial College, London and originally came from Professor Dr. Ralf Bartenschlager. It was used as a positive control and contains replicating plasmid construct with HCV non-structural proteins NS3-NS5B (explained in Chapter 1, section 1.2.2.2). These cells were grown in selective medium containing 750 µg/ml of G418 (selective antibiotic for neomycin resistance gene) (Blight, McKeating et al. 2002). All experiments using replicon cells were carried in the containment level 3 laboratories.

D) Huh 7-J20 cell line

Huh7-J20 cell line is a secreted alkaline phosphatase (SEAP) reporter cell line which was developed by Dr Michaela Iro et al. (Iro, Witteveldt et al. 2009). It stably expresses the enhanced green fluorescent protein (EGFP) fused in-frame with secreted alkaline phosphatase via a recognition sequence of the hepatitis C viral NS3/4A serine protease.

E) THP-1 cell line

Human acute monocytic leukaemia cell line (THP-1 cell line) was grown in 25- or 75- cm³ tissue culture flasks containing Dulbecco's modified eagle medium supplemented with 10% foetal bovine serum.

2.1.2.2. Reagents and other materials

A) Magnetic-activated cell separation columns and CD14

microbeads

MACS separation columns, CD14 microbeads and MACS magnetic separator were purchased from Miltenyl Biotech Ltd UK.

B) MACS buffer

This buffer was formed by adding 0.2mM EDTA and 0.5% heat inactivated foetal calf serum in 500 ml of PBS.

C) Polyethylene glycol 1500

Polyethylene Glycol 1500 was purchased from Roche Biosciences Ltd UK.

D) Primary and secondary antibodies

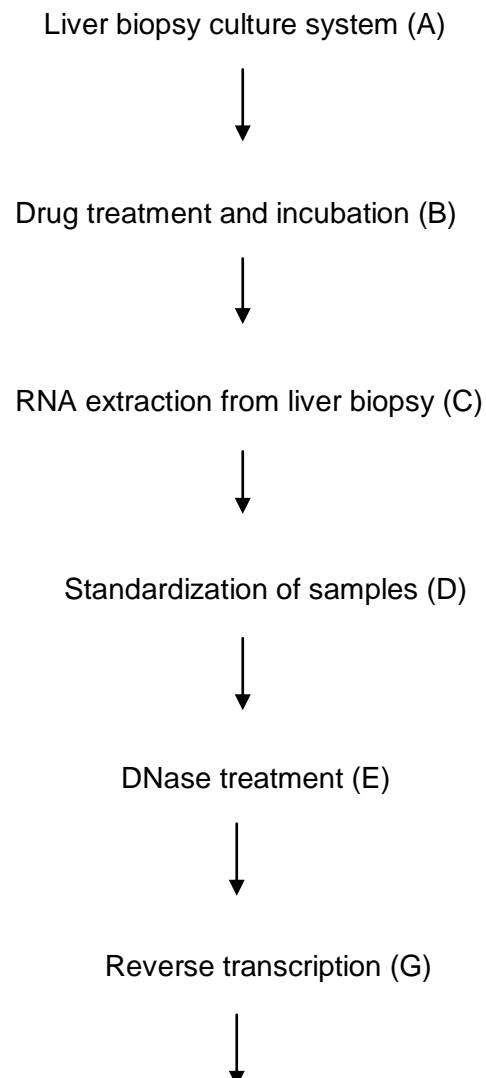
HCV-NS5A was stained with a polyclonal sheep anti-NS5A serum (used at 1/5000 concentration) a kind gift from Professor Mark Harris. Alexa-fluor 488 nm-conjugated IgG goat anti-sheep (from Invitrogen Molecular Probes UK)

was used as secondary antibody against anti-NS5A. Hepatocytes were stained with polyclonal rabbit anti-albumin from DAKO (used at 1/500 concentration) to differentiate them from monocytes. The secondary antibodies were PE-conjugated and alexa-fluor 568 nm-conjugated IgG mouse anti-rabbit (from Invitrogen Molecular Probes UK).

2.2. Methods

2.2.1. Liver biopsy culture model

Overview of experimental steps



Real-time qPCR (H)



Quantification (I)

2.2.1.1. Liver biopsy culture system

Tiny fragments of tissue – approximately 1mm³ excised from the end of the specimen were processed immediately. The fragments were incubated in 500 µl DMEM containing 10% Foetal Calf Serum and 200U/ml penicillin and 100µg/ml streptomycin (Invitrogen UK) along with the appropriate drugs (NA808 at the concentration of 50nM and 500nM, telaprevir at the concentration of 14.7nM (10µg/ml) and IFN-α 2a at the concentration of 500IU/ml). Samples were incubated for 24 and 48 hours at 37°C and 5% CO₂. At the conclusion of incubation cells were isolated from the liver biopsy fragments and were lysed by adding Trizol reagent (Invitrogen) and then stored at -80°C to be analysed later.

2.2.1.2. Cell viability

Cell viability was assessed by an ATP detection assay and by histology at different time points after incubation. Trypan blue dye exclusion method was used as a crude way to determine the number of viable cells in the cell suspension.

a) ATP detection assay

Liver biopsy fragments were dissected and hepatocytes separated out according to the method discussed below. The cells were then used in an ATP detection assay using CellTiter-Glo® Luminescent Cell Viability Assay kit (purchased from Promega, UK) which detects metabolically active, viable cells by quantifying amounts of ATP emitted after addition of a single reagent (CellTiter-Glo® Reagent) directly to cells cultured in serum containing medium after cell lysis. Viable cells are detected within 10 minutes and amount of ATP is directly proportional to the number of cells present in the culture and is detected as luminescent signal (half-life more than 5 hours).

Cell viability of primary human hepatocytes was checked after 24 and 48 hours incubation. Around 1000 hepatocytes in 100µl of culture medium per well were seeded in opaque-walled 96-well plates. 96-well plates were compatible with the luminometer use. Control wells contained medium without cells to obtain a value for background luminescence.

The plate and its contents were equilibrated at room temperature for approximately 30 minutes and a volume of CellTiter-Glo® Reagent equal to the volume of cell culture medium present in each well was added. (i.e.; 100µl of reagent to 100µl of medium containing cells). Contents were mixed for 2 minutes on an orbital shaker to induce cell lysis and the plate was allowed to stand at room temperature for 10 minutes to stabilize luminescent signal. Luminescence was recorded on micro-plate reader - FLUOstar Optima plate reader (BMG Labtech, Aylesbury, UK) with integration time of 1 second per well.

b) Histology of liver biopsy fragments

In experiments performed by Dr Linda Hibbert liver biopsy samples were incubated for 24 hours in DMEM (10% FCS) medium, sectioned in paraffin and stained with haematoxylin and eosin and then analysed histologically.

2.2.1.3. RNA extraction from liver biopsy samples

a) Isolating cells from liver biopsy fragments

Liver biopsy fragments were broken down mechanically by using the plunger of a syringe and pipetting the samples up and down several times. The resulting cell suspension was then passed through a cell strainer to separate out fibrous tissue. Cells were then centrifuged at 500 rpm for 5mins to separate them out from medium and the cell pellet was then homogenised.

The cell density of the cell suspension (in DMEM) was determined using haemocytometer. 0.1 ml of 0.4% trypan blue solution in phosphate buffered saline (PBS) was added to 1 ml of cell suspension and examined under the microscope using haemocytometer. The number of blue staining cells and the number of total cells were counted. Percentage of live cells with intact cell membrane per millilitre was calculated by using the formula:

$$\% \text{ live cells} = [1.00 - (\text{Number of blue cells} / \text{Number of total cells})] \times 100$$

Number of live cells per millilitre was calculated as:

Number of live cells $\times 10^4 \times 1.1 = \text{cells/mL culture}$.

b) Homogenisation

Cell culture medium was discarded, and cells lysed directly by adding 1ml of TriZOL® (trizol) to each well. Trizol is a mono-phasic solution of phenol and guanidine isothiocyanate, it acts by selectively disrupting cells and cell components whilst preserving the integrity of nucleic acids (Wong, Lo et al. 2004). The homogenised cells from each well were transferred to individually labelled eppendorf tubes and stored at -80°C .

c) Phase separation

To each thawed sample 200 μl of chloroform was added; the mixture was then centrifuged at 13000g for 15 minutes at a temperature of 4°C . Total RNA remains in the aqueous phase, protein in the organic phase and the DNA forms a layer between the two. The aqueous phase was transferred to a new tube, and the organic phase stored at -80°C .

d) RNA precipitation

RNA was then recovered from the aqueous phase by addition of 500 μl of cold (-20°C) isopropanol. Each tube was vortexed and then centrifuged at 13000g for 15 minutes at 4°C . This precipitates the RNA in the form of a visible pellet. The isopropanol was removed, and the pellet washed with 1 ml of 75% ethanol, the water component dissolving any remaining salt. The sample was then vortexed and centrifuged at 7500g for 5 minutes at 4°C to

re-precipitate the pellet. The ethanol was then removed and the pellet was allowed to air-dry for approximately 10-20 minutes, the pellet was then dissolved in 40 µl water and warmed at 65°C for 5 minutes for adequate re-suspension.

e) RNA quantification

In order to determine the concentration and the purity of the RNA, a spectrophotometer with full spectrum i.e.; 220-750nm (NanoDrop ND-1000 supplied by Labtech UK) was used. The NanoDrop reader was cleaned with water to prevent contamination and set up to specifically measure RNA. For each sample 2 µl of RNA was placed in the reader and the spectral absorbance at 260 and 280nm was recorded. The RNA yield was noted as a separate reading in ng/µl for every sample. Purity is determined by the ratio of absorbance at 260 and 280nm. Pure RNA is expected to have a ratio of 1.6-1.8 (in DEPC-diethylpyrocarbonate treated water). If the ratio is lower, this indicates protein contamination.

2.2.1.4. Standardisation of samples

The amount of RNA was standardised to 3µg in all samples and genomic DNA was removed by DNase treatment (see below) before the reverse transcriptase (RT) reaction. We standardised all of our samples so that 3µg of total RNA was added to each RT reaction.

2.2.1.5. DNase treatment

Prior to reverse transcription any contaminating genomic DNA was removed by DNase treatment. 3µg samples of RNA were incubated with 4 µl of DNase buffer, and 3 µl of DNase. Samples were incubated for 30 minutes at 37°C in a heating block. After that 4 µl of DNase stop solution was added to each sample and heated at 65°C for 10 minutes to stop the reaction.

2.2.1.6. Principles of reverse transcription

Reverse transcription is the process whereby mRNA is converted into complementary DNA. The reaction is started when oligonucleotide deoxythymine repeats (oligo dt's) bind specifically to mRNA at the poly-adenylated 3-prime tail. Reverse transcriptase, an RNA-dependent DNA-polymerase, then adds specific bases to generate a length of complementary DNA (cDNA). RNAsin is an enzyme which breaks down environmental RNase and prevents degradation of mRNA.

2.2.1.7. Reverse transcription

In the reverse transcription reaction 2 µl of oligo dT₁₅ (from Promega) were added to DNase treated mRNA, and heated at 65°C for 5 minutes. A master-mix was prepared containing the reagents shown in (table 2.3). To each of the sample tubes 24 µl of this master-mix was added. The tubes were placed into a heating block for 60 minutes at 42°C. The cDNA was then stored at -20°C, until the PCR was run.

Constituents of reverse transcriptase master mix

Reagent	Amount added(μ l)
5X1 st Strand Buffer	14
dNTP mix (10mM each dNTP)	2.0
M-MLV Reverse Transcriptase	1.0
RNAasin	1.0
Molecular biology grade H ₂ O	6.0

Table 2.3: Constituents added in the reverse transcriptase master-mix.

2.2.1.8. Real time quantitative polymerase chain reaction

a) Introduction

Polymerase chain reaction (PCR) uses a DNA-polymerase to amplify a piece of DNA by *in vitro* enzymatic replication. As PCR progresses, the DNA generated is itself used as a template for replication. This sets in motion a chain reaction in which the DNA template is exponentially amplified. With PCR it is possible to amplify a single or few copies of a piece of DNA across several orders of magnitude, generating millions more copies of the DNA piece.

Real time qPCR is a highly sensitive and specific technique which enables the amplification and quantification of a specific nucleic acid sequence with detection of the PCR product in real time.

b) Principles of real time qPCR with SYBR® green

During real time PCR either genomic or complementary DNA is incubated with a primer specific for the gene of interest, a fluorescent reporter dye, the DNA-polymerase and single base nucleotides. The reaction mixture is placed into a thermal cycler, in our case the Corbett rotor-gene 6000 (discussed below). The sample is initially heated to 95°C to denature the cDNA and primers ensuring they are single stranded; the temperature is then reduced to the annealing temperature (usually between 50-60°C) allowing the primer to optimally bind the cDNA template; finally the temperature is subsequently increased to 72°C to extend the primer and form the new copy of DNA. This cycle is repeated 45 times and the PCR product accumulates exponentially.

c) SYBR® green

A number of different chemical agents are commercially available to detect newly formed cDNA. SYBR® green is a fluorescent dye that binds double stranded DNA. It emits fluorescent signals of a specific wavelength on binding and excitation with light. Its excitation and emission maxima are 494nm and 521nm, respectively. Detection takes place during extension of PCR product. The signal intensity increases with increasing number of cycles due to accumulation of product. (Illustrated in figure 2.1)

QuantiTect SYBR green PCR master mix (from Qiagen UK) was used for the second step of qPCR, (Constituents of SYBR green master mix are hotStarTaq® DNA Polymerase, QuantiTect SYBR green PCR buffer, dNTP mix, including dUTP, SYBR Green I and 5mM MgCl₂).

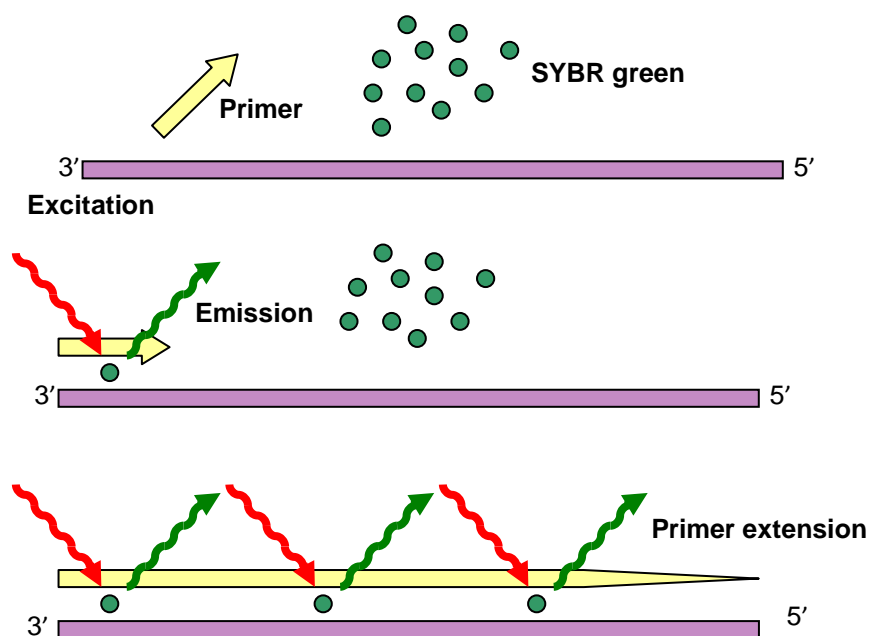


Figure 2.1: Detection of DNA amplification in real time PCR: SYBR® green binds with double stranded DNA and emits fluorescent signal of specific wave length on excitation with light.

d) Corbett rotor-gene 6000

The rotor-gene 6000 (purchased from Qiagen UK) is a real-time cycler that enables highly sensitive real time PCR. It links optical system to thermal cycler and signal is captured at every step of the reaction and provides direct electronic results. Samples spin continuously during a qPCR run at 400 rpm (heating or cooling). High-speed data collection is achieved with all samples read in one revolution (0.15 sec). G-force keeps reagent at the base of tube

which removes bubbles and prevents pellet formation. The rotor-gene can be used in gene expression analysis, pathogen detection, DNA methylation analysis, genotyping, gene scanning and miRNA research.

e) Cycle threshold (C_t)

Signal intensity increases with increase in cycle number because of accumulation of the product. Data is recorded by the rotor gene 6000, and fluorescence plotted against cycle number (illustrated in figure 2.2). For the first 3-15 cycles the fluorescence level is too low to be detected (baseline), once detected the fluorescence increases through the log-linear phase. As all the primer is bound the amount of fluorescence plateaus. The threshold is arbitrarily set to a value above the background and significantly below the plateau of amplification. The cycle threshold (C_T) is the cycle at which the amplification plot crosses the threshold, i.e. at which there is a significant detectable increase in fluorescence. Non-PCR products and primer dimers will also contribute to fluorescence signal. High PCR primer specificity is required when using SYBR® green.

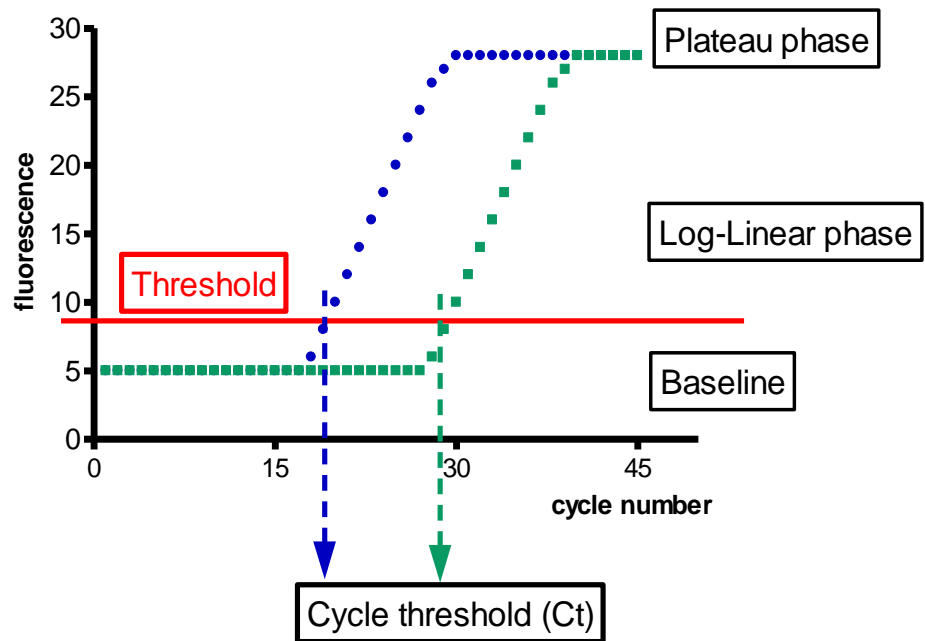


Figure 2.2: Cycle threshold is shown where the amplification plot crosses the threshold, i.e.; at point where there is detectable increase in fluorescence.

f) Polymerase chain reaction

Into each reaction tube the reagents (shown in table 2.4) were added.

A pipetting robot (CAS-1200, Corbett Research) was used to make the master-mixes for the real time qPCR reactions. 72 reaction mixtures could be run at any one time in the rotor-gene.

Constituents of Polymerase Chain Reaction

Reagents	Amount (μl)
2x Quantitect SyBr green mix (Qiagen, UK)	12.5
RNase free H ₂ O (Qiagen, UK)	9.5
Primer mix containing forward and reverse primers (12.5pmol each primer; Sigma-Aldrich)	1
cDNA	2

Table 2.4: Constituents used in polymerase chain reaction

The genes to be amplified were HCV NTR (p1-2 and p3-4), β-actin and MxA.

g) Primers

Primers were obtained from Sigma-Aldrich, sequences and cycling conditions were used according to previously published work (Andonov and Chaudhary 1995, Wehkamp, Harder et al. 2003, Foster, Masri et al. 2004, Thomson, Nastouli et al. 2009) . Sequences and cycling conditions are shown in table 2.5.

Target Gene	Primer sequence	Temperature (°C)			
		Hold 15min	Denaturation 15sec	Annealing 30sec	Extension 20sec
β-actin	Sense 5'CTGGAACGGTGAAGGTGACA 3'	95	95	58	72
	Antisense 5' AAGGGACTTCCTGTAACAATGC 3'				
HCV NTR (p1-2)	Sense 5' AGCGTCTAGCCATGGCGT 3'	95	95	58	72
	Antisense 5' GCACGGTCTACGAGACCT 3'				
HCV NTR (p3-4)	Sense 5' GTGGTCTGCGGAACCGG 3'	95	95	58	72
	Antisense 5' GGGCACTCGCAAGCACCC 3'				
MxA	Sense 5' AACAACTGTGCAGCCAGTA 3'	95	95	58	72
	Antisense 5' AAGGGCAACTCCTGAGAGTG 3'				

2' 5' OAS	Sense				
	5' AACTGCTTCCGACAATCAAC 3'	95	95	58	72
	Antisense				
	5' CCTCCTTCTCCCTCCAAA 3'				

Table 2.5: Sequences and cyclic conditions of primers: HCV NTR (p1-2) (Chen, Lee et al. 2012) and HCV NTR (p3-4) (Harris and Teo 2001), β -actin (Mitsuyoshi, Yasui et al. 2013), MxA (Jones, Davidson et al. 2005) and 2'5' OAS (Dondi, Rogge et al. 2003).

h) Melt curve analysis

Real time confirmation of the specificity of the amplified product is achieved by performing a melt curve analysis. In brief at the end of each PCR run the temperature was increased slowly from a 72°C to 95°C. At low temperatures, all PCR products are double stranded, so SYBR® green binds to them and fluorescence is high, whereas at high temperatures, PCR products are denatured, resulting in rapid decrease in fluorescence. The fluorescence is measured continuously as the temperature is increased and plotted against temperature. A melt curve is produced, because fluorescence decreases slightly through the lower end of the temperature range, but decreases much more rapidly at higher temperatures as the melting temperatures of non-specific and specific PCR products are reached. The detection systems calculate the first derivatives of the curves, resulting in curves with peaks at the respective melt temperatures T_m . Curves with peaks at a T_m lower than

that of the specific PCR product indicate the formation of primer dimers, while diverse peaks with different T_m s or plateaus indicate production of non-specific products. Samples with primer-dimers and non specific products were excluded from subsequent analysis.

i) Samples and controls

In each PCR run samples were run in duplicate, with a no template control; a control reaction that contains all the essential components of the amplification reaction with the template substituted with water, enabling the detection of contamination. In addition a RT minus control was included enabling the detection of amplified genomic DNA. Lastly a positive control sample known to express the genes of interest was included.

j) Quantification of real time qPCR

1) Absolute quantification method using plasmid standards for standard curve generation

In this method, a standard curve was first generated from DNA (obtained from replicon cells) of known concentration for all the genes (β -actin, HCV P1-2, HCV P3-4, MxA and 2'5'OAS) by serial dilution, amplification and cloning into a standard vector. PCR products were generated by using Go-taq ® DNA polymerase (each reaction containing 0.125ul) along with 5xGo-taq buffer (5ul), $MgCl_2$ (2.5ul), dNTP (0.5ul), cDNA or RNase free water in case of negative control (2ul), RNase free water (14.88ul), specific primer (2ul). Template sizes identified through agarose gel electrophoresis are;

- β -actin = 218bp
- HCVP1-2 = 265bp
- HCVp3-4 = 174bp
- MxA = 171bp
- 2'5'OAS = 185bp

PCR products were purified from dNTPs, enzymes and other reaction products using QIAquick PCR purification kit, according to the manufacturer's instructions.

PCR product was cloned into standard vector (pGEM[®]-T vector – size 3000bp, from Promega) according to the protocol given with pGEM[®]-T Easy vector system. These plasmids were later transformed into the bacteria (DH5a E.Coli cells) and the bacterial colonies grown on agar plates at 37°C. Colonies were then picked up and added to reaction tubes containing Go-taq[®] DNA polymerase (each reaction containing 0.125 μ l) along with 5xGo-taq green buffer (5 μ l), MgCl₂ (2.5ul), dNTP (0.5ul, RNase free water (14.88 μ l), specific primers (2 μ l) and agarose gel electrophoresis was performed to chose the colonies containing cloned plasmids. Colonies with appropriate bands on gel electrophoresis were grown overnight and plasmid DNA was purified from overnight culture of E.Coli in LB (Luria-Bertani) medium by using QIAprep spin miniprep kit according to the manufacturer's instructions (some of these cultured samples were mixed with 50% glycerol and stocked in -80 ° C prior to purification). Purified plasmid products were amplified by PCR and sent for sequence analysis to Geneservice Ltd UK. Further plasmid containing bacterial colonies from glycerol stocks were grown overnight in 500 ml and further purified by using Qiagen plasmid midi and maxi kits

according to protocol provided with the kit. These samples were then diluted in 1:1000 to form DNA standards.

Spectrophotometric measurements at 260/280 nm were used to assess the concentration of these DNAs, which were then converted to a copy number value based on the molecular weight of the sample used. The following formula was used:

$$\text{Xg/ } \mu\text{l DNA} / [\text{plasmid length in base pairs} \times 660] \times 6.022 \times 10^{23} = \text{Y molecules/ } \mu\text{l}$$

cDNA plasmids are the preferred standards for standard curve quantitation. However, since cDNA plasmids don't control for variations in the efficiency of the reverse transcription step, this method only provides information on relative changes in mRNA expression. This, and variation introduced due to variable RNA inputs, was corrected by normalization to a housekeeping gene (β -actin).

After calculating the number plasmid molecules with the target viral gene in a microlitre, a series of dilutions was made to generate a PCR standard curve. Though a time consuming process, the use of absolutely quantified DNA standards helps to generate absolute copy number data from unknown samples.

2) Comparative C_T method

'Comparative CT method' to quantify HCV RNA was also used parallel to the above method. This method involved comparing the CT values of the

samples of interest with a reference or calibrator such as a non-treated sample in our case. The CT values of both the calibrator and the samples of interest were normalized to an appropriate endogenous housekeeping gene (β -actin).

k) Agarose gel electrophoresis

This method was carried out to confirm that the amplified product was of the expected size. A ladder of fragments of DNA of a known size was used as a marker. 3 g of agarose was mixed with 200 ml 0.5X TBE (Tris borate EDTA) buffer. This mixture was micro-waved until the agarose had fully dissolved. Once cooled, 3 μ l of ethidium bromide was added and gently mixed. The agarose was poured into the gel tray and the comb positioned. This was allowed to set. 5 μ l 6X PCR loading buffer (Promega) was added to each PCR reaction tube. The comb was removed and the gel was placed into a tank of 0.5X TBE running buffer. The wells were washed with the running buffer to remove any extra agarose. 20 μ l of the sample / buffer mix were loaded into the wells. 5 μ l of the 100bp DNA marker (Promega; also premixed with loading buffer) was added to one well. The electrodes were attached and the gel was run at 100 volts until the bromophenol blue dye was approximately 4cm from the bottom of the gel. The PCR bands were visualized by placing the gel on an ultraviolet light box.

l) Statistical analysis

Each sample was run in duplicate during PCR and the averages calculated. Differences in gene expression between treated and non treated samples

were analysed using the Wilcoxon matched pair test. The statistical software package Prism 5, was used to prepare graphs and perform the statistical analyses.

2.2.2. Cell-to-cell fusion experiments

All experiments were carried out in containment level 3 laboratories.

2.2.2.1. Separation of PBMCs from blood (density gradient separation)

Blood from the patients (within 4 hours of venesection) was taken into preservative free heparin-100 units per 10 ml of blood, and diluted with equal amount of Dulbecco's modified eagle medium. 15 ml of ficoll-Paque™ PLUS (GE Healthcare, Buckinghamshire, UK) and ficoll was mixed thoroughly before use and was added to 50 ml conical tube and 35 ml diluted blood was layered carefully on ficoll. After layering the blood over ficoll the tube was spun in a bench centrifuge at 2000 rpm for 20 minutes at 20°C, with brakes off. The upper plasma layer was removed and discarded without disturbing the plasma-ficoll interface. Mononuclear cells make a layer at the interface between the two liquids and red cells (and majority of polymorphs) go to the bottom of the tube. The mononuclear cell layer was transferred into a fresh tube, taking this entire layer but with the minimum of the ficoll and tissue culture medium layers. The mononuclear cells were diluted with tissue culture medium and spun at 1500 rpm for 10 minutes. The medium was removed from the cell pellet and the cells were washed twice. These cells

were resuspended in a known volume before the last wash and taking a small aliquot for counting using a haemocytometer. The cells were then plated over night onto a 75 cm³ flask to allow adherence. Adherent cells were separated out from the 75 cm³ flask using a cell scraper, the following day.

Note: Ficoll, blood and medium were kept at room temperature (15 - 25°C) for optimum results. Principle of separation-- Differences in cell density is exploited to separate granulocytes and erythrocytes from mononuclear cells. Granulocytes and erythrocytes have a higher density at the osmotic pressure of ficoll, and they sediment through the ficoll layer during centrifugation. Ficoll-paque™ PLUS also enhances erythrocyte aggregation to increase erythrocyte sedimentation through the ficoll layer. Mononuclear cells, with lower densities, remain at the plasma-ficoll interface.

2.2.2.2. Magnetic-activated cell separation (MACS) of monocytes

1. Principle: positive selection

Cells are magnetically labelled with CD14 microbeads (purchased from Miltenyl Biotech Ltd, Surrey, UK) and separated on a column which is placed in the magnetic field of a MACS separator. The magnetically labelled CD14+ cells are retained in the column and the unlabelled CD14 -ve cells run through as they are depleted of CD14 +ve cells. After removal of the column from the magnetic field, the magnetically retained CD14 +ve cells are eluted as positively selected cell fraction.

2. Steps of MACS separation

A) Magnetic labelling

Monocytes were magnetically labelled with

CD14 microbeads



Magnetic Labelling

B) Magnetic separation

Cells are separated in a MACS column placed

in a MACS separator. The flow-through fraction

can be collected as negative fraction depleted

of labelled cells.



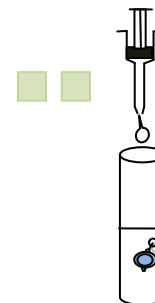
Magnetic separation

C) Elution of the labelled cell fraction

The column is removed from the separator.

The retained cells are eluted as the enriched

positively selected cell fraction.



Elution of the labeled cells

D) Sample preparation

Platelets are removed after density-gradient

separation by resuspending the cells in MACS

buffer and rotating in a centrifuge at 1500 rpm

Figure 2.3: Steps to separate monocytes by MACS separation method

for 10-15 minutes at 20°C.

a) Magnetic labelling (using CD14 microbeads)

The PBMCs were kept on ice to prevent capping of antibodies on the cell surface and non-specific labelling with CD14 microbeads (Miltenyl Biotech, UK) which occurs at higher temperature. PBMCs isolated from 40mls of blood usually yielded $2.5 \times 10^{-7} \pm 0.5 \times 10^{-7}$. They were resuspended in MACS buffer (PBS containing 0.5% FCS + 2mM EDTA—kept at 2-8°C) at a ratio of 80 µl of buffer per 10^{-7} cells. 20 µl of CD14 microbeads were added per 10^{-7} cells, mixed well and incubated for 15 minutes in the refrigerator (2-8°C). Cells were then washed by adding 1-2 ml of MACS buffer per 10^{-7} cells and centrifuged at 1500 rpm for 10 minutes. Supernatant was aspirated completely and cells were resuspended in 500µl of MACS buffer.

b) Magnetic separation by MS columns

For the above number of PBMCs, MS column (Miltenyl Biotech Ltd UK) was selected. The column was placed in magnetic field of MiniMACS Separator (Miltenyl Biotech UK). MS column was prepared by rinsing with 500 µl of MACS buffer and cell suspension applied onto the column. Unlabelled cells passing through were discarded and the column washed three times with 500 µl of MACS buffer. Column was removed from the separator and placed on a 15 ml conical tube. 1 ml of buffer was poured onto the column and magnetically labelled CD14 +ve monocytes were immediately flushed out by firmly pushing the plunger into the column. Monocytes were counted using

haemocytometer. A total of 6×10^6 monocytes were isolated from 40 ml blood.

2.2.2.3. PEG fusion of Huh 7.5s and monocytes

1) Aims of fusion experiments

- To determine optimal fusion conditions.
- To estimate the percentage of infected fused cells and whether HCV could be propagated into the Huh 7.5 cells after fusing them with HCV infected monocytes.
- To determine how long the Hepatitis C virus can be detected in the fused cells.

2) Use of dye labelled monocytes and Huh 7.5

A small proportion of cells were labelled with colour dyes (Vybrant™ cell labelling solutions—Dil-PE and DiO-FITC (purchased from Invitrogen Molecular Probes) before fusion. The Dil-PE dye generates red fluorescence in the FACs scanner whilst the DiO-FITC dye fluoresces green. Separate cells can therefore be distinguished from fused cells by simple colour analysis. Some Huh 7.5s were labelled with DiO and some were labelled with Dil dye and left unfused to act as controls for flowcytometry. Other Huh 7.5s were labelled with DiO and fused with Dil labelled monocytes to estimate the degree and amount of fusion which was done by colour sorting (Flowcytometry). This labelling was done so that fusion could be analysed by Flowcytometry (FACS). Cells were washed thrice, centrifuged at 1200 rpm

for 10 min each time and fixed with 4% paraformaldehyde. Cells were reconstituted in FACS buffer (1%BSA/10%FCS in PBS). FACS sorting was carried out by the flowcytomter, BD LSR II analyser (BD Biosciences). Data were analysed using FlowJo software, version 7.6.5 (TreeStar, Ashland, OR).

a) Method used for dye labelling

2×10^{-6} to 4×10^{-6} of monocytes (depending upon the numbers isolated) and Huh7.5 cells each were suspended in 1 ml of serum free medium in two eppendorf tubes. 5 μ l of vybrant Dil-labelling solution were added to 1 ml of monocyte containing medium and 5 μ l of vybrant DiO-labelling solution were added to 1 ml of huh 7.5 containing medium. Gentle pipetting was done for even distribution of dyes. Tubes were then kept at 37°C for 20 minutes to allow efficient cell labelling. After 20 minutes the two tubes with labelled cells were centrifuged at 1200 rpm for 5 minutes and washed thrice with serum free medium. Both cell populations were transferred to a 15 ml conical tube and centrifuged so that they pelleted together at the bottom of tube. The supernatant was discarded and tube spun again and extra medium discarded.

b) Fusion of dye labelled fused cells

The dye labelled cells were treated with 500 μ l of polyethylene glycol (PEG-1500 from Roche Diagnostics, Burgess Hill, UK) already incubated at 37°C and stirred for even distribution and kept in the incubator. After 3 minutes this mixture was treated with tissue culture medium (37°C). Note that the time taken from the addition of PEG to the addition of medium was kept to a

maximum of 3 minutes. This time is important to bring the cell membranes together and allows fusion to occur without significant cell damage from the exposure of PEG. While putting the medium, first millilitre was added drop by drop in 1 minute so that the cell hybrids were not disrupted and rest of the 4 ml were added slowly in 2 min. These cells were then centrifuged at 1500 rpm for 5 min and were harvested in 4% paraformaldehyde to be analysed by flowcytometry and percentage of fusion was assessed. Fusion efficiency was checked at different days too. Some fused cells were grown on 24 well plates and isolated on later days with CD14 microbeads and again analysed with flowcytometry after fixing with 4% paraformaldehyde. This was done to see whether fusion persisted afterwards in these cells or not. These dye labelled fused cells were also used in our experiments to optimize the method of fusion.

c) Use of dye labelled cells in optimizing fusion

We looked at few things to optimize our method of fusion. We looked at the amount of time needed to keep the cells in PEG 1500 for more efficient fusion to take place and to see how maximum number of cells can fuse together without too much cell death due to PEG 1500 toxicity. In order to find out what ratio of both monocytes and Huh 7.5 cells will give us the maximum numbers of fused cells we tried different proportions of monocytes and Huh 7.5 cells to find out optimal ratio. Dye labelled cells were used (explained above) in these experiments.

To determine the optimal time required for effective fusion isolated monocytes were labelled with Dil and mixed with equal numbers of DiO labelled Huh 7.5 cells (Huh 7.5 cells were labelled with DiO and Dil separately to be used as controls during the flowcytometry analysis). The mixture was separated in three tubes. In each tube fusion was done in the same way with PEG 1500 but the only difference was time in the incubator before adding the tissue culture medium. Cells were grown and fixed after 24 hours with 4 % paraformaldehyde and analysed with flowcytometry to check which time was most favourable for fusion. Some cells were extracted at day 3 to be tested by real time PCR for HCV expression. To estimate the optimal ratios of both monocytes and Huh 7.5 cells to be used in fusion we fused different ratios of both monocytes and Huh 7.5 cells i.e. 1:2, 1:1 and 2:1. We used PEG 1500 with different pHs to determine the relationship between the amount of fusion with the pH of PEG 1500.

d) Unlabelled fused cells

Having established an optimal procedure for fusing hepatocytes and CD14 bead separated monocytes, we proceeded to experiments in which we fused monocytes from treatment naïve chronic hepatitis C patients with Huh 7.5 cells and looked at the HCV expression in fused cells at different times. Cells were fused without labelling with colour dyes and grown for different time periods. The fused cells were seeded into 6 well plates at a density of 5×10^5 cells/mL and maintained at 37°C. Real time polymerase chain reaction and indirect immunofluorescence (both confocal and non-confocal) microscopy were used for HCV detection and replication analysis.

2.2.2.4. Fusion for HCV detection and replication

2×10^{-6} to 4×10^{-6} monocytes and huh 7.5 cells, both in 1:1 ratio and unlabeled were fused by PEG 1500 in the same way as mentioned above and the time taken from the addition of PEG 1500 to the addition of medium was 3 minutes. Fused cells were resuspended in 5 ml of medium added slowly as mentioned above. 500 μ l of this suspension was incubated in each well of a 12 well plate and grown for the indicated time points, ideally 3, 5 and 7 days, although longer time periods were tried to determine the extent of HCV replication. The cells were passaged when they became confluent. Fused cells were processed – RNA was extracted in trizol reagent as previously described. Real time PCR was used to detect HCV expression. In few fusion experiments the fused cells were bead selected for CD14 at different time periods and were grown on cover slips for immunofluorescence microscopy to confirm HCV replication in fused cells.

2.2.2.5. CD14 microbeads separation of fused cells

Cells were CD14 microbead separated to analyse subpopulations of Huh 7.5 cells fused with monocytes and to select out cells which are more likely to express HCV RNA. These cells were stained with anti-NS5A antibody to detect HCV. The cells were also labelled with anti-albumin stain to localise HCV replication in Huh 7.5 cells. Albumin expression is a feature of hepatocytes and hepatic cell lines.

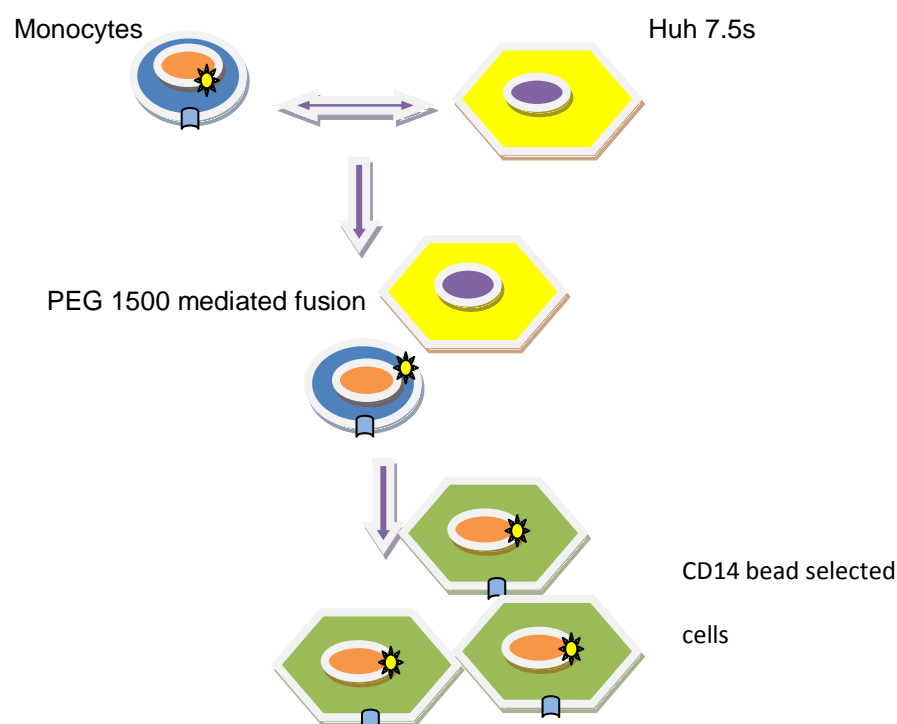


Figure 2.4: This figure shows the steps used for PEG 1500 fusion and then CD14 bead separation to isolate CD14 labelled cells.

2.2.2.6. Capture transfer assay

Monocytes were isolated from blood of a healthy donor and incubated with serum of a hepatitis C infected patient containing high titres of HCV for 24 hours before fusion. PEG 1500 mediated fusion was done with Huh 7.5s in the similar way mentioned above. The cells were grown on cover slips after CD14 bead separation and stained to look for the presence of HCV replication in fused cell.

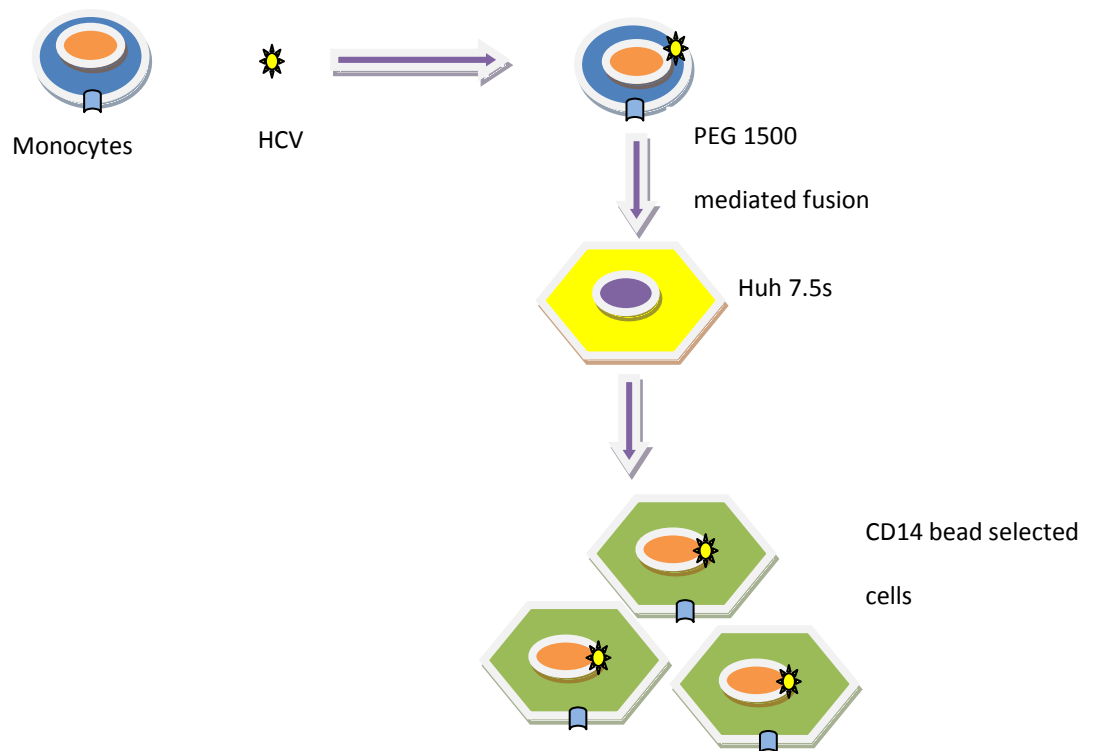


Figure 2.5: Monocytes isolated from healthy donor were fused with Huh 7.5 cells after incubating them in HCV patient's serum for 24 hours and CD14 bead separated.

2.2.2.7. Quantification of HCV expression

HCV expression was quantified by real-time quantitative polymerase chain reaction. (See chapter 2 for method)

2.2.2.8. Indirect immunofluorescence microscopy

13 mm Cover slips were first coated with polyethyleneimine for 1 hour at room temperature and rinsed well with sterile water thrice each for 5 minutes. Cover slips were allowed to dry completely and sterilized under UV light for 4 hours. Cells were grown on glass cover slips and rinsed briefly in phosphate-buffered saline (PBS).

a) Fixation

Cells were fixed in ice-cold methanol, acetone (1-10 minutes) or in 3-4% paraformaldehyde in PBS with pH of 7.4 for 15-20 min at room temperature and washed thrice with ice cold PBS (5mins/wash).

b) Permeabilization

Cells were then permeabilized (Note: acetone fixed samples do not require permeabilization). Samples were incubated for 10 minutes with PBS containing 0.25% Triton X-100 (Note: Triton X-100 is the most popular detergent for increasing penetration of the antibody. However, it is not appropriate to be used for membrane associated antigens since it destroys membranes). Cells were washed in PBS three times for 5 min.

c) Blocking and incubation

Cells were incubated with 10% Foetal Bovine Serum or in some cases 10% serum from the specie that the secondary antibody was raised in, for 30 minutes to block unspecific binding of the antibodies. Anti-NS5A was added in the above blocking buffer in humidified chamber for 1 hr at room temperature (in dark) / or overnight at 4°C. Cells were then washed with PBS Tween20 0.02% thrice for 5 minutes each wash in dark. The secondary antibody was used in similar way. Anti-albumin staining was then done in the similar way with secondary antibody following the primary antibody.

d) Counter staining and mounting

Cells were mounted with Vector shield with DAPI – hard set purchased from Vectral Laboratories ltd UK and left for 30 minutes at room temperature in dark and looked at under an immunofluorescence microscope.

e) Immunofluorescence microscopy

Labelled fused cells with replicon containing cells taken as positive control and unfused Huh 7.5 cells as negative control were viewed on a Leica DM5000 automated epifluorescence microscope (Leica Microsystems, Milton Keynes, UK, www.Leicamicrosystems.com) and camera used to capture images was MM Leica – QIClick Qimaging 12 bit Monochrome, 1.4 Megapixels. Images were viewed under oil-immersion lenses x40 and x63 to detect NS5A and albumin staining.

f) Confocal microscopy

Confocal immunofluorescence microscopy enables the reconstruction of three dimensional structures. By the mean of a confocal pinhole, the microscope is efficient at rejecting 'out of focus' fluorescent light resulting in image coming from a thin section of the sample and it scans thin sections through the sample resulting in formation of a very clean three dimensional image of the sample.

For confocal microscopy fused cells along with negative and positive controls were viewed under Andor Technology Revolution DSD Spinning disk (Andor

Technology, Belfast, UK www.andor.com) and Zeiss LSM510 inverted microscope META detector laser (Carl Zeiss, Welwyn Garden City, UK www.zeiss.com) confocal microscope under an oil-immersion lens of x63 objective with numerical aperture of 1.40. Alexa-fluor 488 antibody was excited using an argon laser fitted with 488 nm filter in Zeiss 510 META and 488 nm 30mW laser lines in Spinning disk. Alexa-fluor 568 antibody was excited using the argon laser which was fitted with 561 nm 30mW filter in Spinning disk. Vybrant dye Dil (orange—red-fluorescent dye, which is spectrally similar to PE) was excited with the 543 helium / neon laser line while vybrant dye DiO (green-fluorescent dye, which is spectrally similar to FITC) was excited with the 488 nm argon laser line in Zeiss 510 META. DAPI was excited with 405 filters in both Zeiss 510 META and Spinning disk. Images are given in the result section and are displayed as single optical sections of 40 μm thickness.

2.2.2.9. Fusion with SEAP reported cell line

As mentioned earlier SEAP reporter cell line expresses the enhanced green fluorescent protein (EGFP) fused in-frame with secreted alkaline phosphatase via a recognition sequence of the hepatitis C viral NS3/4A serine protease. The main principle of SEAP reporter cell line is that it secretes alkaline phosphatase in culture medium if it gets infected with hepatitis C virus because of cleavage at NS3/4A serine protease site by the virus leading to secretion of alkaline phosphatase fused with EGFP. This leads to production of luminescence which can be detected by luminometer. We fused 4 patients' monocytes (infected with different genotypes strain of

virus) with SEAP reporter cell line (Huh 7.5 J20 cells) and grew fused cells for 1 to 4 days. We incubated some cells with telaprevir while others were kept as untreated controls. At day 1 and day 4 we took off medium from the top of these cells and used this medium to detect for the presence of alkaline phosphatase by using Great EscAPe[™] SEAP Chemiluminescence Kit 2.0 as per manufacturer's instructions (placental alkaline phosphatase was used in different dilutions, i.e. 1:10 and 1:100, as positive control and was provided with the kit) and detected luminescence by using a 96-well micro-plate reader (FLUOstar OPTIMA micro-plate reader with integration time of 1 second) to detect and record SEAP signal.

2.2.3. Formation of immunosuppressed cell line

2.2.3.1. Plasmid construction - 'pEF PIV5-V IRES.neo'

As discussed in Chapter 1, section 1.7.5, V protein of parainfluenza virus 5 (PIV5) forms either direct or indirect complexes with STAT1 and is known to block IFN signalling by targeting STAT1 for proteasome mediated degradation and inhibits the action of IFN. We speculated HCV replication will increase in absence of IFN induced host cellular response and transfected Huh 7.5 cells with PIV5 plasmid to impair IFN induced signalling and ISGs production.

Plasmid construct 'pEF PIV5-V IRES.neo' was a kind gift from Professor Richard Randall, University of St. Andrews. It is a 7556 bases containing plasmid with EcoR1 site at position 1 destroyed, rendering a unique site in the multiple cloning sites. The construct 'pEF.PIV5-V' has been cloned in

'pEF.IRES.neo' (6374bp) between NcoI and NotI sites. Other elements included in plasmid are residual SV40 fragment (at 16-99bp), EF-1 promoter (at 99 to 1291bp), R-globin 5'UTR (at 1291-1349bp), Polio IRES (at 1405-2044bp), Neomycin resistance (at 2044-2954bp), R-globin 3'UTR/polyA (at 2954-3730bp), ampicillin resistance ORF (at 5385-6780bp). NcoI sites are at 1350bp, 2981bp and 3835bp. NotI site is at 2530bp. Restriction enzymes NcoI and NotI were purchased from Promega UK.

2.2.3.2. Transformation of bacteria

E.Coli DH5 α bacterial cells (purchased from Invitrogen UK) were used for transformation. About 10-20 μ g of each plasmid DNA was used per 30-50 μ l DH5 α competent cells (thawed on ice) for cloning. After mixing plasmid DNA and bacterial competent cells and incubating them on ice for 30 minutes they were heat shocked at 42 $^{\circ}$ C for 50 seconds and left on ice for 2 minutes. 250 μ l S.O.C medium (purchased from Invitrogen UK) was added in the above mixture and incubated for 1 hour at 37 $^{\circ}$ C. 50 μ l from the above mixture was then spread on plates containing Lysogeny broth (LB) agar (mixed with ampicillin). It was then allowed to dry for 10 minutes and incubated at 37 $^{\circ}$ C (plates inverted) overnight. Ampicillin selected colonies were grown overnight.

2.2.3.3. Plasmid purification

The plasmid purification from bacterial cells was carried out using **EndoFree Plasmid Maxi Kit** (Purchased from Qiagen UK). Single colony was picked up from every plate and a starter culture of 2-5ml Lysogeny Broth (LB) medium

containing ampicillin was inoculated for every plasmid and incubated for 8 hours at 37°C with vigorous shaking (at the rate of 300 rpm). This culture was diluted to 100 ml and grown at 37°C for 12-16h with vigorous shaking. If the culture showed a considerable growth, bacterial cells were harvested by centrifugation at 6000xg for 15 minutes at 4°C. The pelleted cells were separated out after supernatant was removed and were resuspended completely by vortexing in 10 ml RNase A containing Buffer P1 (50mM Tris·Cl, pH 8.0, 10mM EDTA;) until no cell clumps remained. 10ml Buffer P2 (200mM NaOH, 1% SDS (w/v)) was added and mixed gently but thoroughly by inverting 4-6 times, and incubated at room temperature for 5 minutes. During this time QIAfilter Maxi Cartridge was prepared by screwing a cap onto the outlet nozzle and placing it into a 50ml Falcon tube. 10ml chilled Buffer P3 (3.0 M potassium acetate, pH 5.5) was added to above lysate and mixed immediately but gently by inverting 4-6 times and poured into the barrel of QIAfilter Maxi Cartridge and incubated at room temperature for 10 minutes. The cap from the QIAfilter Maxi Cartridge outlet nozzle was removed and plunger was inserted gently into the QIAfilter Maxi Cartridge and cell lysate was filtered into 50ml tubes. Buffer ER (Composition not given) was added to the above filtered lysate and mixed by inverting the tube approximately 10 times and incubated on ice for 30 minutes. QIAGEN-tip 500 was equilibrated by applying 10ml Buffer QBT (750 mM NaCl; 50 mM MOPS, pH 7.0; 15% isopropanol (v/v); 0.15% Triton® X-100 (v/v)) and allowed to empty by gravity flow. Filtered lysate was applied to the equilibrated QIAGEN-tip and allowed to enter the resin by gravity flow and QIAGEN-tip washed with 30 ml Buffer QC (1.0 M NaCl; 50 mM MOPS, pH 7.0; 15%

isopropanol (v/v)) twice) and DNA eluted with 15 ml Buffer QN (1.6 M NaCl; 50 mM MOPS, pH 7.0; 15% isopropanol (v/v)). DNA was precipitated by adding 10.5 ml isopropanol at room temperature to eluted DNA and centrifuged immediately at 15000xg for 30 minutes at 4°C. Supernatant was carefully decanted off. DNA pellets were washed with 70% ethanol and centrifuged at 15000xg for 10 minutes and supernatant was carefully decanted off and pellet was air dried for 5-10 minutes and dissolved in 500 µl endotoxin-free water supplied with the kit.

2.2.3.4. Quantification of plasmid DNA

In order to determine the concentration and the purity of the plasmid, a spectrophotometer with full spectrum i.e.; 220-750nm (NanoDrop ND-1000 supplied by Labtech UK) was used. The NanoDrop reader was cleaned with water to prevent contamination and set up to specifically measure DNA. For each sample 2 µl of DNA was placed in the reader and the spectral absorbance at 260 and 280nm was recorded. The DNA yield was noted as a separate reading in ng/µl for every plasmid. Purity is determined by the ratio of absorbance at 260 and 280nm. Pure DNA is expected to have a ratio of 1.6-1.8 (In DEPC-diethylpyrocarbonate treated water). If the ratio is lower, this indicates protein or phenol contamination.

2.2.3.5. Restriction enzyme digestion of plasmid

pCI-Puro^R and pCI-B14R plasmids were digested with EcoR-1 and Xba-1 restriction endonucleases separately to get controls and also with both

enzymes together to calculate appropriate band size. PIV5-V-IRES.neo plasmid was digested with Nco-1 and Not-1 enzymes separately and also with both restriction enzymes together. The enzymes digestion mixes contained 3 µl buffer (NEBuffer 4 was used for Ecor-1 and Xba-1 and NEBuffer 3 was used for Nco-1 and Not-1) (New England Biolab, UK), 1 µl restriction enzymes (New England Biolab, UK), 0.5 µl BSA, 1µg plasmid DNA and diluted in DEPC treated water to make up to 30 µl in total.

2.2.3.6. Agarose gel electrophoresis to detect band size

After restriction enzyme digestion of plasmids we detected the band sizes with agarose gel electrophoresis. The plasmids with right inserts were sent for sequencing to Gene Service UK.

2.2.3.7. Transfection in Huh 7.5 cells

Cells were plated out at a density of 0.5×10^6 /well of a 6 well plate 6 hours before the transfection. This was to ensure that cells are in log phase of their growth when the transfection was done. Two concentrations of DNA were used for transfections - 5 and 10 µg. Cells were either transfected with single plasmid or with two plasmids together as shown in figure 2.6. Plasmid DNA was diluted with OptiMEM (Invitrogen UK) to 100 µl for each well. At the same time 5 µl Eugene (Invitrogen UK) was diluted to 100 µl with OptiMEM for each well. These two mixes were then left at room temperature for 40 minutes and then mixed together for 15 minutes at room temperature. Wells of 6 well plates were washed with OptiMEM twice and 600 µl was added in each well. Above transfection mixes was added in each well and incubated

at 37°C for 1.5 hours. 3 ml DMEM (10% FCS) was added in the above mixture and incubated for 24 hours. 3 ml of G418 400 µg/ml containing DMEM (10% FCS) was added after 24 hours in each well and cells were left to grow for 4-6 weeks and antibody selection medium was changed after 2-3 days. After 4-6 weeks cells started growing in PIV5-IRES.neo (5 µg) plasmid containing well. The cells were then shifted in a 75 mm³ flask when confluent. At all subsequent passages antibody selected medium was used.

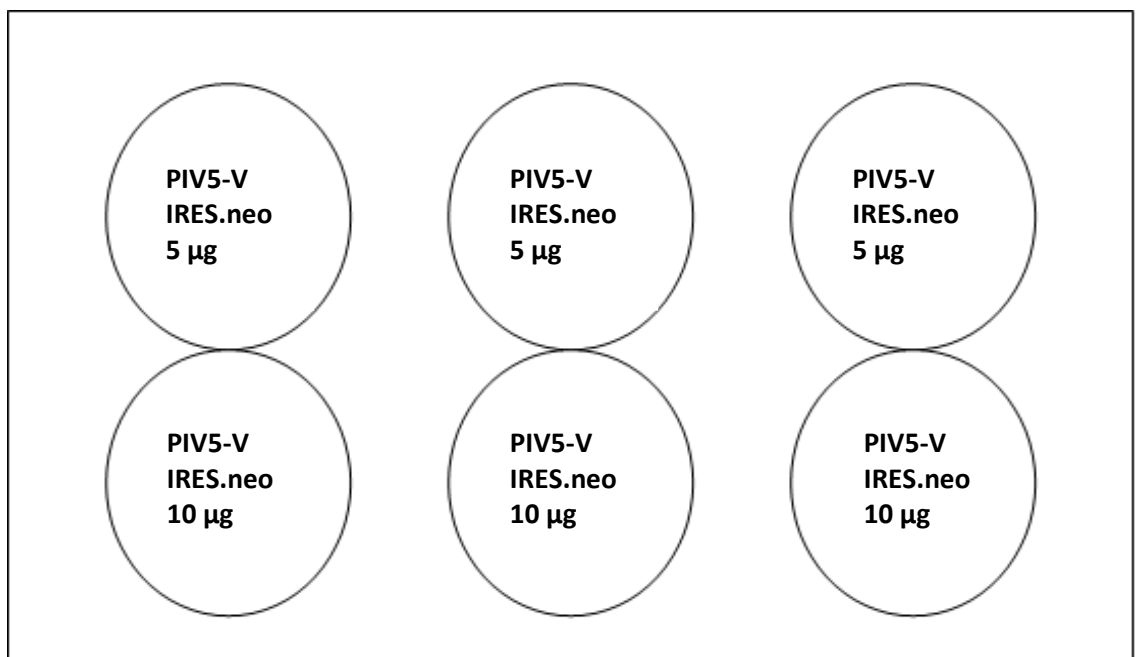


Figure 2.6: Transfections in 6 well plate with the plasmid (PIV5-V IRES.neo) at 5 and 10µg concentrations.

Chapter Three

***Ex Vivo* Liver Biopsy Assay of Hepatitis C Virus**

3.1. Introduction

Development of *in vitro* systems such as sub-genomic replicating HCV (HCV replicon) or models involving unusual strains of genotype 2 HCV (JFH-1) have facilitated our understanding of the dynamics of chronic HCV infection (Lohmann, Korner et al. 1999). However, these replicon cell lines display deregulated gene expression, abnormal proliferation, aberrant signalling and endocytic function. The HCV replicon depends on tissue culture adaptive mutations exists only for a few genotypes and may not represent human virus. Moreover, JFH-1 has unusual features (reviewed in Bartenschlager and Sparacio 2007) and is limited to the non-structural gene of a genotype 2a HCV strain derived from a single patient. These issues with *in vitro* models necessitate the use of animal models, either chimpanzees or mice with engrafted human livers, to study more typical strains of HCV. A robust *ex vivo* or *in vitro* infectious system with clinical isolates of heterogeneous HCV genomes in a given patient does not exist and alternative systems allowing preclinical efficacy testing of novel antiviral compounds are required.

Many drugs inhibit viral replication by interacting with viral enzymes. Telaprevir is one such drug, which is now licensed for clinical use. It inhibits the viral protease (HCV NS3-4A protease) which is involved in cleavage of nascent proteins used for final assembly of new virions. In one study chimpanzees infected by HCV clones with defective NS3-4A function did not show HCV infectivity and it is thought that NS3-4A protease has a role in HCV polyprotein cleavage, maturation and viral replication (reviewed in Kolykhalov, Mihalik et al. 2000). NS3-4A protease is also implicated in

evasion of host immune responses through down regulation of signalling molecules involved in production of IFNs (Foy, Li et al. 2003, Li, Foy et al. 2005). Drugs such as telaprevir (VX-950) and NA808 are used to evaluate HCV replication in the liver biopsy model in this chapter. Studies have shown that telaprevir is more potent against the genotype 1 strain of HCV (Reesink, Zeuzem et al. 2006) and lowers HCV RNA during the initial 2 days of monotherapy. However, emergence of resistant mutants is a significant problem in later days if the drug is used in isolation (Forestier, Reesink et al. 2007). NA808 is an exploratory drug developed by 'Chugai Pharmaceuticals' by screening a drug library using genotype 1 replicon cells. This agent is considered a potent inhibitor of HCV replication and appears to act on cellular targets; although the precise mechanism of its antiviral effect is unknown at this time experiments by the company involving HCV patients suggested that the drug was safe. However, when experiments in man were initiated, it showed no evidence of antiviral activity at concentrations that were non toxic and this compound was therefore withdrawn from further development.

A previous study suggests that incubating liver biopsies with interferon allows a prediction of therapeutic responses (Chang, Sung et al. 2009). Preliminary work in our lab by Dr Linda Hibbert suggested that fragments of human liver biopsies could be used to support HCV replication for a short period of time and this work extends these studies to see if a model system could be developed and whether pre-clinical drug testing could be possible with this model. Thus, to allow drug testing on naturally occurring virus isolated from patients an *ex vivo* culture assay of liver fragments isolated from biopsies

performed routinely on chronic HCV patients was used. This might allow testing of novel antiviral compounds on diverse HCV genomes of clinically relevant genotypes and might be used to analyse dynamic changes in HCV gene expression after exposure to drugs.

The liver is a heterogeneous organ containing hepatocytes, fibroblasts, endothelial cells, stellate cells and Kuffer cells (Cho, Park et al. 2010). There are cell-to-cell and cell-matrix interactions which provide adequate conditions and cell signalling for tissue modulation, cell-migration, differentiation and growth (Cho, Berthiaume et al. 2008, van Poll, Parekkadan et al. 2008, Cho, Park et al. 2010). This microenvironment and external stimuli are crucial in the proper function, autonomy and survival of cells. The microenvironment includes adjacent cells, extracellular matrix, growth factors, local forces and soluble stimuli (Ploss, Khetani et al. 2010). Primary hepatocytes in 'blocks' of tissue (as in our intact liver biopsy fragment model) have advantages over other human liver models as they retain the integrity of their cytoarchitecture (Sivaraman, Leach et al. 2005, Hewitt, Lechon et al. 2007, Khetani and Bhatia 2008). In contrast, when hepatocytes are cultured alone they disintegrate, undergo karyolysis and lose cytoskeleton structure. (Bhatia, Balis et al. 1999). Experiments (outlined below in section 3.3.2) show that cell viability is rapidly reduced in hepatocytes that have been extracted from their surrounding matrix.

To improve the *in vitro* environment of liver cells attempts have been made to co-culture them with other cells to provide some of the matrix/cellular interactions that may be important for maintaining hepatocyte function. These

co-culturing techniques are used to provide a favourable microenvironment for adequate cell functioning and to achieve maximum benefits by controlling and modulating the degree of contact (Bhatia, Balis et al. 1999, Khetani and Bhatia 2008, Ploss, Khetani et al. 2010).

In recent years micro-patterning techniques have been used to provide organotypic cultures. Studies have shown that hepatocyte survival and function improve if they are grown in a specific 2 dimensional, co-planar format with 3T3-J2 embryonic fibroblasts (Bhatia, Balis et al. 1999, Khetani and Bhatia 2008, Ploss, Khetani et al. 2010). This co-culture technique has recently gained popularity and has a central role in monitoring drug response, metabolism and toxicity. This provides a platform for cell-to-cell interaction. The model described in these studies involves a miniaturized model with spherical islands of primary human hepatocytes co-cultivated with 3T3-J2 murine embryonic fibroblasts to induce heterotypic and homotypic synergy of cells. This interdependence is vital for preservation of hepatocyte phenotype and function over prolonged periods as cells make polarized layers with optimized dimensions crucial for maintaining viability and liver specific function. The micro-patterning model to maintain hepatocyte functions involving 3T3-J2 murine embryonic fibroblasts has allowed hepatocytes to maintain albumin production, urea synthesis, glycogen storage and E-cadherin expression over many weeks. They retain inducibility of cytochrome P450 and phase II enzymes and, importantly, have been shown to support HCV replication, albeit in a very transient, low level infection system (Bhatia, Balis et al. 1999, Khetani and Bhatia 2008, Ploss, Khetani et al. 2010). The micro-patterning approach may provide an optimal environment for

maintaining hepatocytes in culture. However, the system is technically very challenging and has not been reproduced outside the initiator's laboratory.

Real time quantitative PCR analysis for quantification of HCV RNA in liver biopsy model was used as our primary approach for HCV RNA quantification. RNA extracted from liver tissue was subjected to reverse transcription (RT) and specific domains in UTR region of cDNA obtained from RT were amplified. RNA was measured by UV absorbance at 260 nm and 280 nm and this is based on RNA being absorbed at 260nm and proteins at 280 nm. RNA purity is tested as the 260 nm/280 nm ratio while lower ratios indicate contamination. In order to stabilise RNA it is reconstituted in DEPC (Diethyl-pyrocabonate) treated water or in Tris solution (2-amino-2-hydroxymethyl-1,3-propandiol). In one study, RNA isolated from mouse liver, was tested for influence of dissolving in both solutions discussed above. It was found that 260/280 absorbance ratio of RNA dissolved in distilled water was around 1.82 ± 0.01 (n=5) while that dissolved in DEPC-treated water and Tris were 1.52 ± 0.01 (n=5) and >2.17 (n=5) respectively. DEPC-treated water did not seem to affect the absorbance at 260 nm, but elevated absorbance at 280 nm while Tris-HCl (1 M, pH 7.0 or 10.0) lowered the absorbance at 260 nm and even more at 280 nm thus, the 260 nm/280nm ratio was elevated. These results demonstrate that the measuring solution has an impact on the overall absorbance ratio of RNA. RNA absorbed in Tris might falsely show increased RNA purity and DEPC treated water might under represent the purity of RNA. If 260/280 absorbance ratio of RNA is 1.6 to 1.8 when reconstituted in DEPC treated water, it is acceptable because DEPC treated

water has PH of 5 and will give a lower absorbance ratio (Wilfinger, Mackey et al. 1997).

Moreover, in another study RNA Absorbance at 260/280 was different at different pH. At higher pH of solvent RNA absorbance ratio rises over-representing the RNA purity while at lower pH RNA absorbance appears lower even if RNA is free of protein and may under-represents the ratio by 0.2-0.3 (Wilfinger, Mackey et al. 1997, Okamoto and Okabe 2000).

The sample preparation method and time taken to extract RNA from time of sample collection can impact on RNA quality and concentration and liver biopsy fragments are processed after 24-48h of collection. DEPC water was used to prevent contamination with RNases (degrade RNA). DEPC inactivates RNase through covalent modification. In our experiments we used RNase free 0.1%DEPC treated water/autoclaved. There is no RNA extraction method which yields RNA completely free of genomic DNA so enzymatic DNA removal of RNA sample is recommended. RNA was decontaminated from genomic DNA by DNase treatment. In order to prevent sample degradation extensive freeze/thaw cycle was avoided by aliquoting samples. Filtered pipette tips were used to minimise contamination between samples. RNA was stored in siliconized tubes to avoid adsorption of RNA with the walls of tubes.

For PCR primers amplifying short products are known to give higher PCR efficiency. Therefore, the primers chosen for our experiments had lengths

between 18-30 nts. GC contents was between 45-60%, (GC content of 30% is minimum, 50% is optimal and 80% is maximum) (D'Haene, Vandesompele et al. 2010). Primers not containing runs of >3 G or C nucleotides were used. Sequence complementarities between forward and reverse primers were avoided and a BLAST search was performed to ensure specificity, amplification efficiencies were calculated based upon the generation of standard curves using plasmid DNA dilution series. Melt curve analysis and gel electrophoresis was used to gauge specificity of PCR reactions. In order to prevent secondary structures G/C rich areas and palindromes were avoided.

3.2. Aims and background

Development of novel therapies for chronic hepatitis C virus (HCV) infection can potentially be streamlined by using patient-derived model systems in which drugs can be tested prior to establishing clinical trials. Such models have been hampered by difficulties in maintaining primary human hepatocytes from infected individuals in culture. This chapter looks at the use of surplus tissue fragments of routine biopsy samples from patients with HCV to develop an *ex vivo* system for antiviral drug testing by incubating these samples for a definite time in laboratory and allowing estimation of HCV gene expression in response to antiviral drugs.

To improve the value of the assay it was speculated that separating the hepatocytes from the biopsy fragment and culturing as isolated cells may reduce sample to sample variability, by increasing the homogeneity of the cells. It was postulated that growing the PHHs in contact with other stromal

cells (such as 3T3 cells) might allow survival of the cells for sufficient length of time to allow assessment of antiviral drugs. The aim of this research was to develop simple, easy to use culture conditions that could be used to maintain differentiated hepatocytes in culture for several days to allow studies on HCV replication to be completed. These experiments are based upon the micro-patterning model but in view of the complexity of this technique aim was to develop a simpler system, but at the expense of lesser robustness, that would facilitate future studies of HCV replication.

3.3. Results

3.3.1. Set up experiments for HCV detection

A) Developing a PCR based assay for HCV RNA detection – assay involving ‘replicon’ containing cells.

Different numbers of replicon cells i.e.; 3×10^{-6} , 2×10^{-6} , 1×10^{-6} , 0.5×10^{-6} , 0.25×10^{-6} and 0.1×10^{-6} cells were used to obtain different concentrations of mRNA (determined using the Nanodrop reader (see Methods)). mRNA concentrations extracted from these cells were 3355.8ng/μl (1.87), 2824.3ng/μl (1.95), 805.6ng/μl (1.95), 508.3ng/μl (1.87), 152.2ng/μl (1.82), and 84ng/μl (1.70) respectively (with absorbance ratios at 260/280 in brackets) were obtained. A total amount of RNA of 3μg was taken from each sample, using larger aliquots from samples with lower RNA concentrations compared to samples with higher concentrations, to generate equal amounts of cDNA from all samples. Real time quantitative PCR was used to detect gene expression of β-actin and HCV p1-2. C_T values of all samples were

within 3-4 cycles for both β -actin and HCV p1-2 (real time qPCR plots with melt curves are shown in figure 3.1 and 3.2 for β -actin and HCV p1-2 respectively).

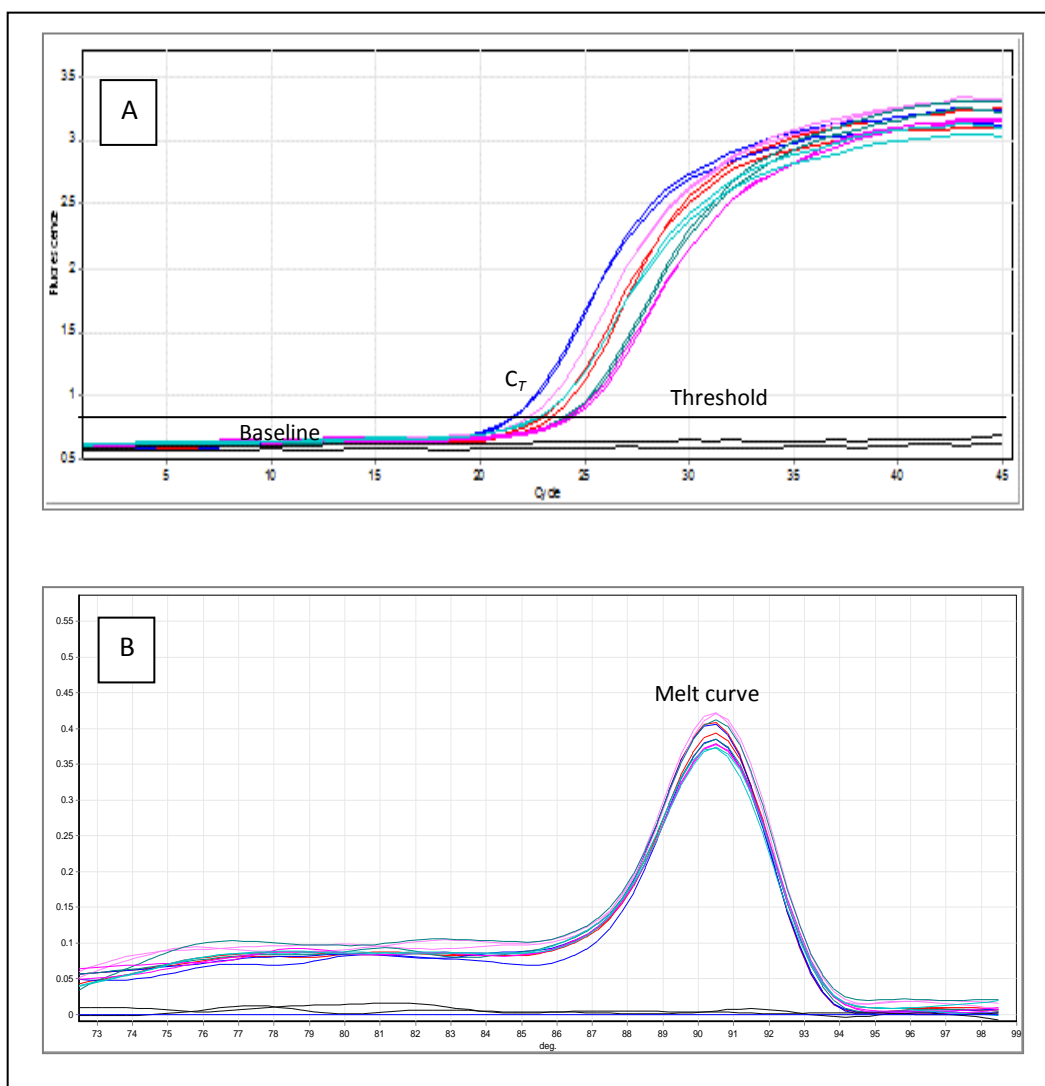


Figure 3.1: Real time qPCR plot and melt curve analysis of β -actin: A) PCR plot with C_T values (for β -actin) of each sample ran in duplicate and difference of no more than 3 cycle lengths between two different samples (range of C_T between samples is 18.93 - 21.85). B) Melt curve of β -actin shows a melt temperature- T_m in range of 90-91°C.

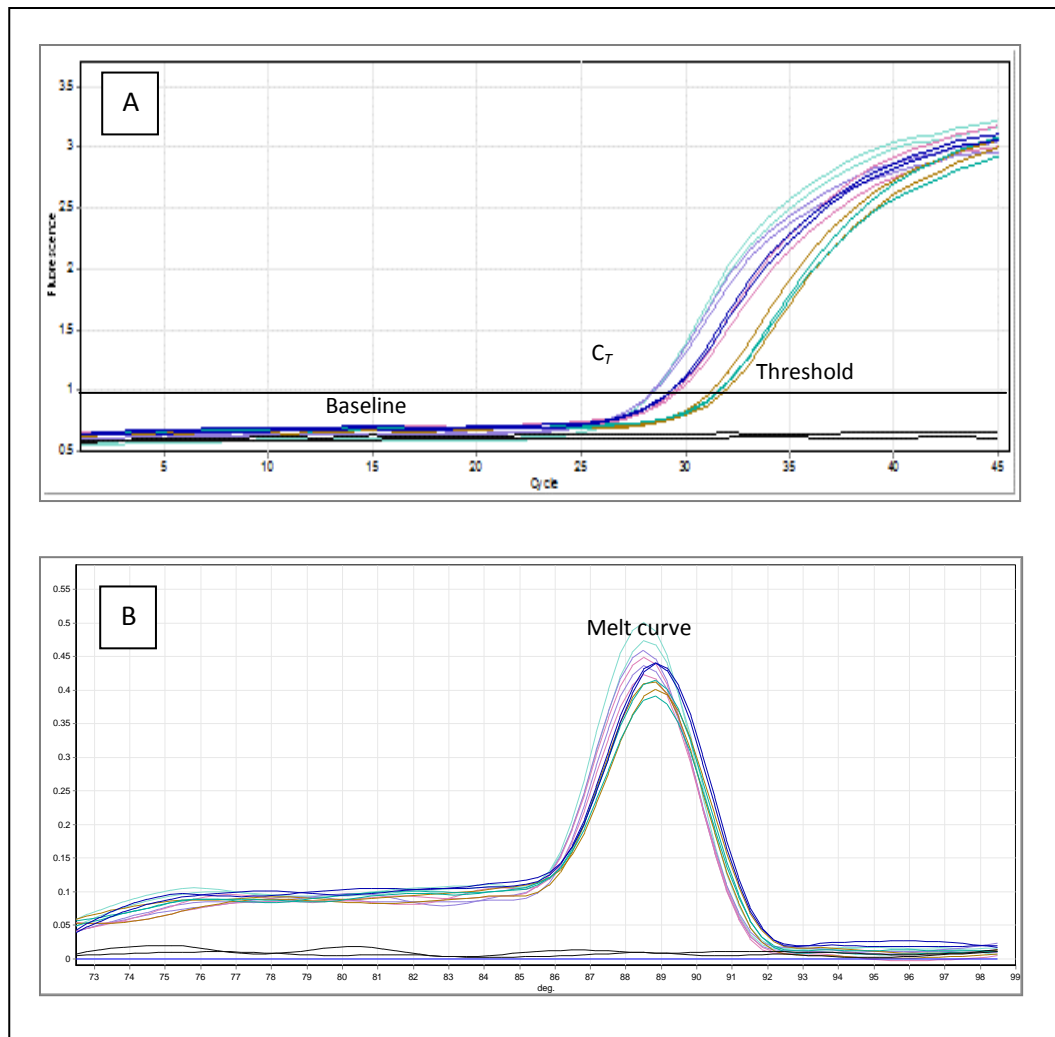


Figure 3.2: Real time PCR plot and melt curve analysis of HCVp1-2: A) PCR plot with C_T values (HCVp1-2) of each sample ran in duplicate and difference of no more than 4 cycle lengths between two different samples (range of C_T between samples is 24.49 - 28.25). B) Melt curve of HCVp1-2 shows a melt temperature- T_m in range of 88-89°C.

These experiments show that the real time qPCR assay robustly detects HCV RNA from different cell numbers in a replication system with little variability. These studies indicate that the difference in the initial cell number should not affect the results. Hence, the HCV/ β -actin ratio is the same whatever the starting number of cells.

B) Limit of detection of HCV gene expression

In order to estimate the limit of detection of HCV RNA gene expression measured through real time qPCR, serial dilutions of replicon cells were achieved by using Huh 7.5 cells to obtain different ratios of infected and uninfected cells. Total 1×10^6 cells were used in each sample with different concentrations of both Huh 7.5 cells and replicon cells. The number of replicon cells in the dilution series were 1×10^6 , 0.5×10^6 , 0.25×10^6 , 0.12×10^6 , 62500, 31250 cells respectively. HCV RNA gene expression was analysed by real time qPCR and relative quantification technique (discussed in chapter 2 section 2.2.1.8). HCV gene expression (normalised to β -actin) was calculated in all samples. The average HCV expression (expressed as $2^{-\Delta\Delta C_T}$) was 0.005 (range 0.01 to 0.003, $n=1$) and all samples were positive. This study showed that HCV RNA could be detected in infected cell counts as small as 31250 cells ($2^{-\Delta\Delta C_T} = 0.003$). As the average number of cells obtained from the liver biopsy fragments was around 50000 cells in each sample with a high probability of many cells being infected we speculated that our assay had a reasonable chance of detecting HCV RNA in human liver.

C) Polymerase chain reaction efficiency

i) Primers

T_m values of primers were calculated by using this formula: $(2^\circ\text{C} \times (A+T) + 4^\circ\text{C} \times (G+C))$. Melt curve analysis was carried out as discussed above, to

prevent non-specific PCR products or primer dimerisation (5%DMSO added to prevent primer dimer formation).

ii) Standard curve for primer efficiency

1×10^{-6} Replicon cells were used to extract cDNA and DNA plasmid standards were generated through technique discussed in chapter 2 section 2.2.1.8. After spectrophotometric determination of plasmid DNA concentration, the copy number of standard DNA molecule was calculated using the formula:

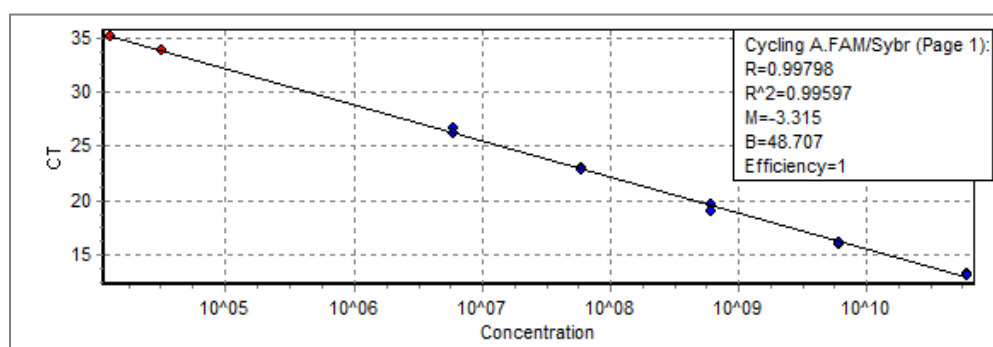
Copy numbers of DNA molecule = $(x \text{ g}/\mu\text{l DNA} / [\text{plasmid length in base pairs} \times 660]) \times 6.022 \times 10^{23} = Y \text{ molecules}/\mu\text{l}$

Plasmid length = vector (pGEM® T vector) length + template size

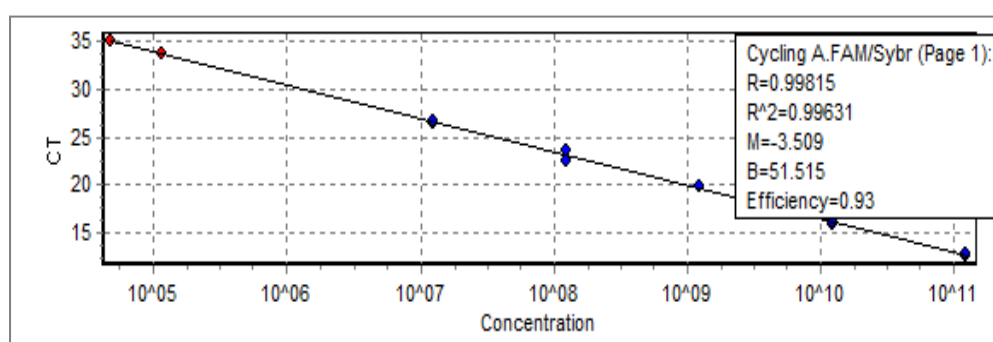
Template sizes are given in chapter 2, section 2.2.1.8. Plasmid DNA concentrations were: β -actin = 213ng/ μl , HCVp1-2 = 440ng/ μl , HCVp3-4 = 265.5ng/ μl , MxA = 329ng/ μl , 2'5'OAS = 222ng/ μl . Copy numbers of DNA plasmid standards after 1:1000 dilutions were calculated as: β -actin = 6.04×10^7 , HCVp1-2 = 1.23×10^8 , HCVp3-4 = 7.61×10^7 , MxA = 9.46×10^7 , 2'5'OAS = 6.64×10^7 .

The efficiency of PCR was evaluated by performing a dilution series experiment using the target assay and creating a standard curve (using plasmid DNA standards) using different genes (primers: β -actin, HCVp1-2, HCVp3-4, MxA and 2'5'OAS). Real time qPCR with SYBR® green was used to detect gene expression. Rotorgene 6000 software was used to generate standard curves. The slopes (M) of the standard curves were translated into

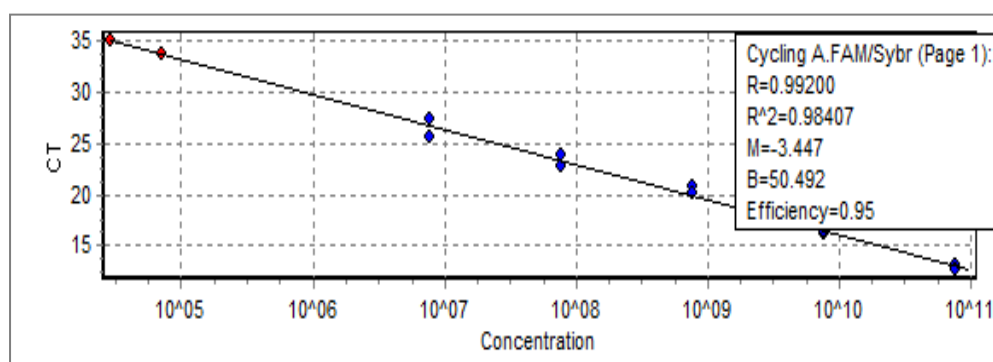
efficiency value (Efficiency (E) = $10^{-1/M}$). Standard curves with slopes, efficiencies and R² values are shown in Figure 3.3.



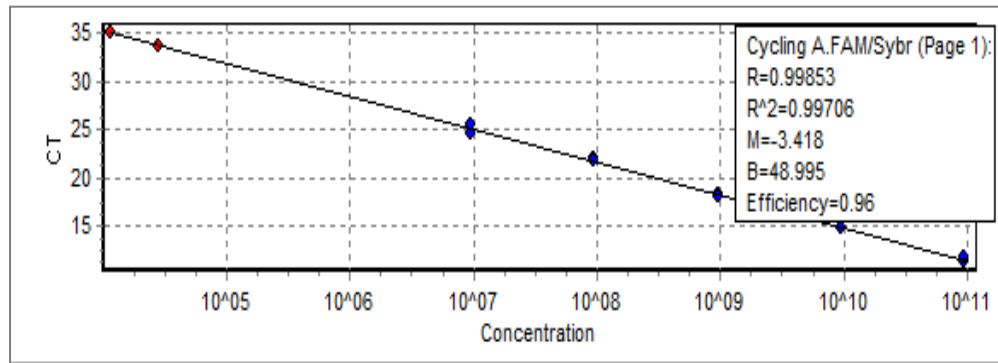
Standard curve for β-actin



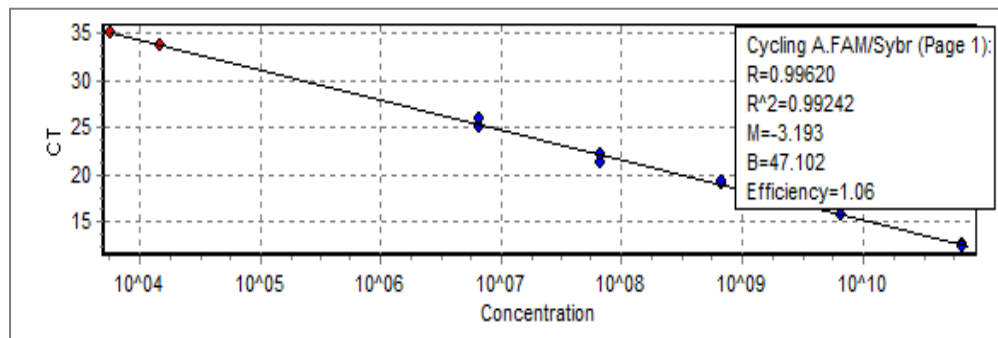
Standard curve for HCVP1-2



Standard curve for HCVP3-4



Standard curve for MxA



Standard curve for 2'5'OAS

Figure 3.3: Standard curves created from dilution series of the plasmid DNA: The R^2 value is a measure of the fit of the data points to the regressed line. The slope (M in the equation) is an indication of the PCR efficiency. A slope of -3.3 indicates 100% PCR efficiency (i.e., the number of copies of amplification product is doubled at each cycle). The y intercept (b in the equation) is defined as the y value (C_t) when x (log concentration) equals 0.

If a standard curve has a correlation coefficient (R^2 value) < 0.99 , the data accuracy becomes less predictable. Rotor gene 6000 software was used to perform a linear regression to the standard dilution series data and to calculate the equation for the best-fit line (the standard curve). The equation is in the form of $y = Mx + B$, where $x = \log \text{ concentration}$ and $y = C_t$.

Therefore, B gives the C_T for the 1ng/ μ l standard if concentrations are defined as ($\log_{10}(1) = 0$). The slope M is indicative of the PCR efficiency. A slope of -3.3 indicates 100% (1.00) PCR efficiency (i.e., the number of copies of amplification product is doubled at each cycle).

The slopes created by our standard curves range from -3.2 (efficiency 1.06) to -3.5 (efficiency 0.93).

iii) Amplification plots

Dilution series of plasmid standards with known target were used to form amplification plots.

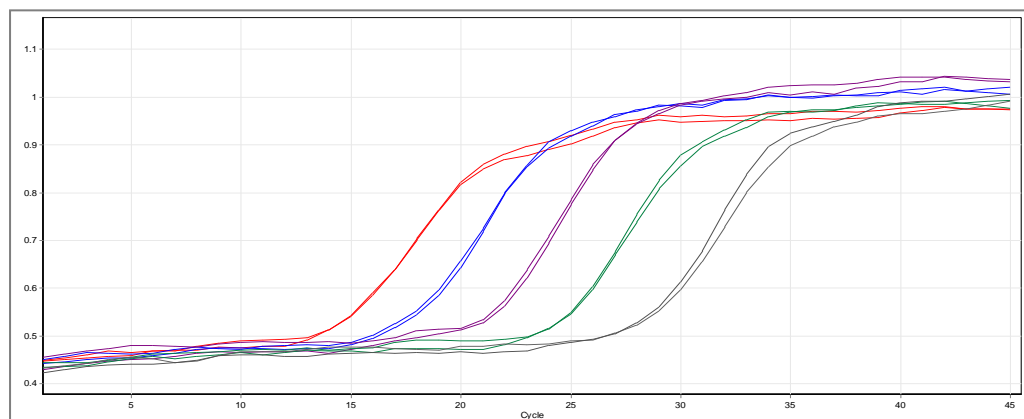


Figure 3.4: Dilution series (1:10) of the target gene - β -actin, amplified in duplicate.

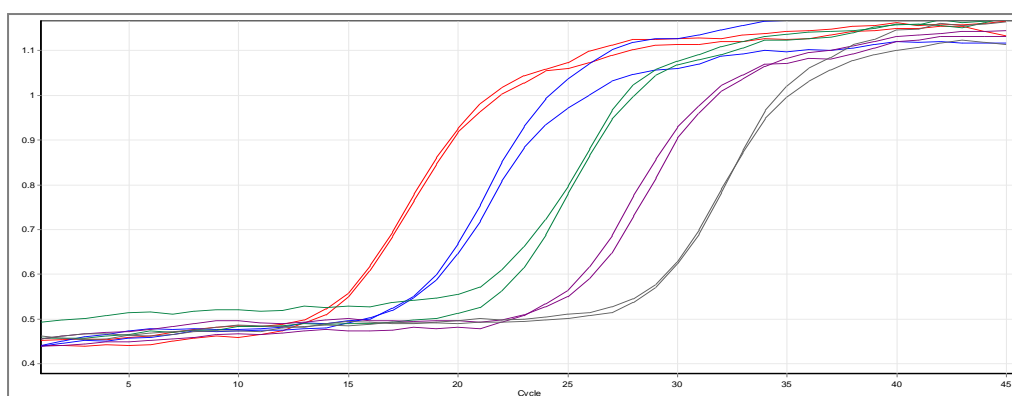


Figure 3.5: Dilution series (1:10) of the target gene - HCVp1-2, amplified in duplicate.

During the initial set up of real time qPCR it was essential to include identical replicates for each input cDNA. The use of these replicates helps in gauging the accuracy of the results especially at lower C_T values. If identical replicates have a C_T value of >0.3 and/or a standard curve has a correlation coefficient (R^2 value) <0.99 then results are less precise. However, it is important to know that at higher C_T values (34-40) with low fold change in target there could be increased C_T variation among replicates due to statistical distribution and this unavoidably gives rise to poorer precision. Thus the difference between C_T values of identical replicates was less than 0.3 at lower C_T values while at values above 34-40 difference was less than 0.5.

iv) RNA quantification

Quality and yield of RNAs obtained from all liver biopsy samples studied were estimated by spectrophotometer with full spectrum i.e.; 220-750nm

(NanoDrop ND-1000 supplied by Labtech UK, discussed in Chapter 2 method section 2.2.1.3).

Nanodrop readings (RNA concentration expressed in ng/μl and absorbance at 260/280) for all samples derived from liver biopsies were accumulated and plotted as histograms. The RNA yield was plotted in ng/μl for every sample (see figure 3.6).

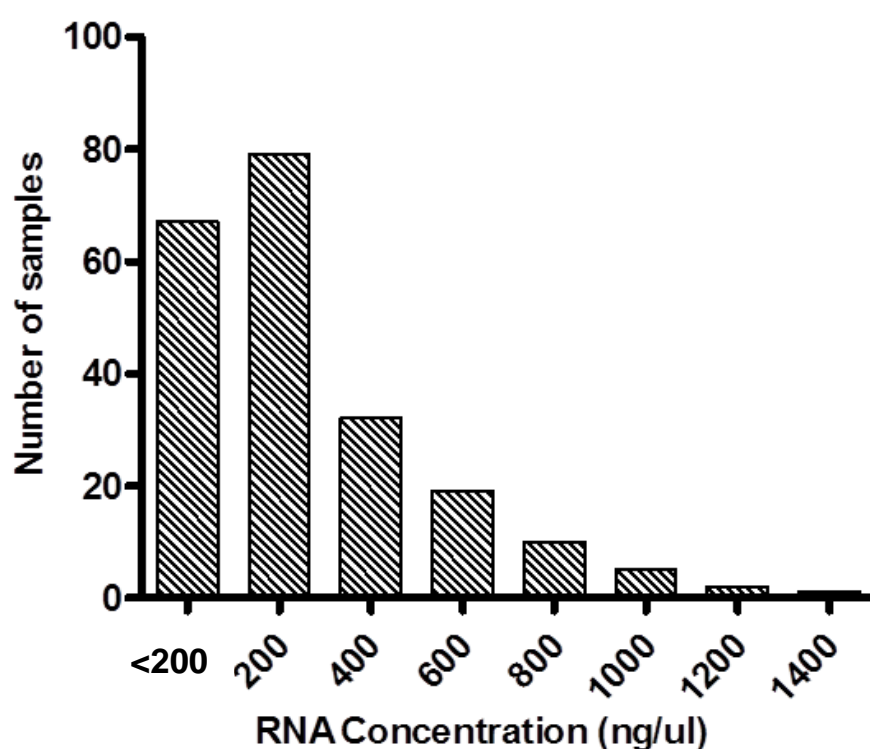


Figure 3.6: RNA concentration measured by the nanodrop (spectrophotometer): The RNA concentration was more than 100ng/ul in more than 89% of samples.

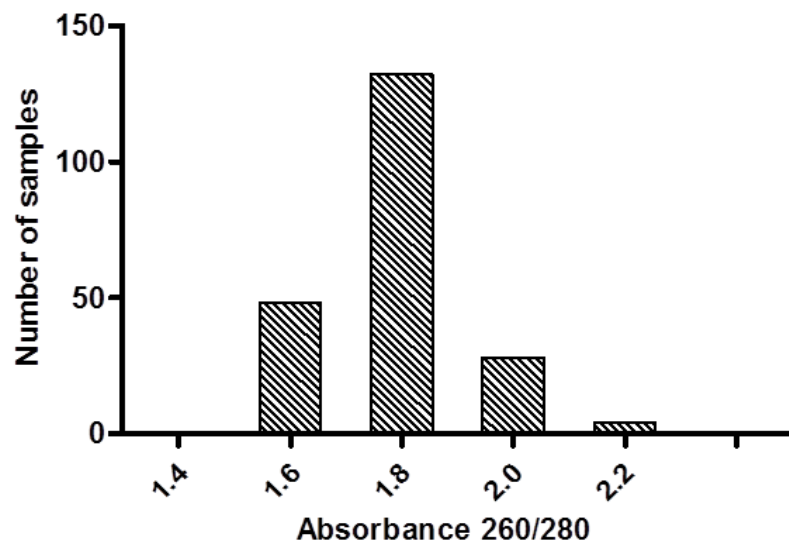


Figure 3.7: RNA quality determined by comparing ratio of spectral absorbance at 260nm and 280nm: More than 71% samples had 260/280 absorbance ratio in range of 1.8-2.0. RNA samples in DEPC treated water could give lower 260/280 absorbance ratio due to acidic pH of DEPC treated water (Wilfinger, Mackey et al. 1997, Okamoto and Okabe 2000).

v) Quantification of polymerase chain reaction

Target nucleic acid quantification can be carried out using two techniques; absolute quantification and relative quantification. Absolute quantification is carried out by using set standards with known concentration or copy number. These standards contain the same sequence as that of the target sequence or differ only slightly from target nucleic acid. A standard curve is a measure of plot of C_T values divided by crossing points of different dilutions against the log of the amount of the standard and is generated by using a dilution series of the standards. The initial amount of target used for PCR

amplification is quantified against the known standards by its comparing C_T value with known standards. Relative quantification calculates the ratio between the amounts of target nucleic acid with a reference gene (i.e.; housekeeping gene) in the same sample and this normalised value is used to compare gene expressions in difference samples. Relative quantification of gene expression quantifies differences in the expression level of a specific target (gene) between different samples. The data output is expressed as a fold-change or a fold-difference of the expression levels. In absolute quantification a reference standard containing target gene into a plasmid was constructed and a calibrated curve was created by using known amount of plasmid and amount of target was calculated against this as an absolute term as number of copies (Li and Wang, 2000). Absolute quantification however relies on highly accurate dsDNA quantification and construction of reliable standard curves. It requires adequate pipetting as standard curve requires dilution over several orders of magnitude. qPCR results were analyzed by two types of relative quantification methods. In one method HCV infection was expressed as gene expression calibrated to β -actin while the other method was delta-delta C_T method in which we calibrated HCV gene expression against β -actin and normalized with the positive control (cells containing HCV replicon). It was observed that the trend was exactly the same with both the methods. Results here are given by the first method and expressed as HCV/ β -actin ratio.

vi) Validation of housekeeping gene

Previous experiments performed in our laboratory by Dr Linda Hibbert compared HCV RNA to β -actin (HKG used in mRNA levels with HCV RNA to GAPDH mRNA levels and HCV RNA to transthyretin mRNA (a hepatocyte specific gene) in order to determine whether alternative 'normalising' mRNAs were associated with different results in RNA from four different patients (two genotype 1, one genotype 3 and one genotype 4). No significant changes in sample to sample variation were seen when the data was normalised to housekeeping genes other than β -actin (personal communication Dr Linda Hibbert). Endogenous housekeeping genes are used to normalize differences in the amount of cDNA that is used into a PCR reaction. Endogenous control expression levels must be the same in all samples under all conditions in both control and experimental group. Real time qPCR can be used to test uniform expression of endogenous controls by comparing their C_T levels in control and experimental groups by using equivalent volumes of the cDNA for PCR reactions. HKG expression level must not change under experimental conditions. In order to perform the comparative C_T method (also known as the $\Delta\Delta C_T$ method) the target and endogenous control should have relatively equivalent PCR efficiencies.

3.3.2. Viability of liver biopsy fragments

Viability of liver biopsies was assessed by different methods.

3.3.2.1. Histology of liver biopsy fragments

In experiments performed by Dr Linda Hibbert liver biopsy samples were incubated for 24 hours in the medium and then analysed histologically. Figure 3.8 shows results from these experiments and indicates that liver morphology is maintained in the biopsy fragments after 24 hours incubation.

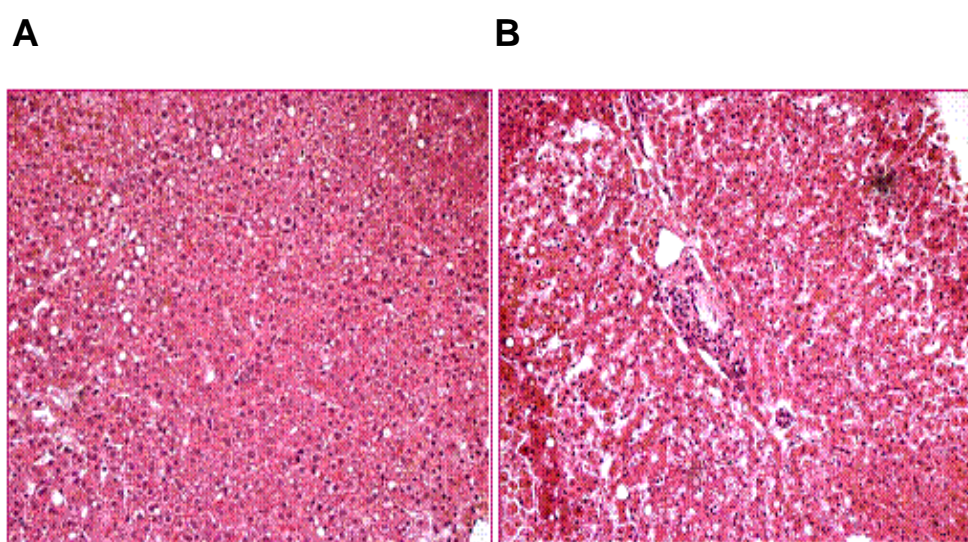


Figure 3.8: Histological demonstration of the tissue viability: (A&B) Haematoxylin and eosin stained sections from the same liver biopsy specimen at (A) baseline and (B) after 24h culture in DMEM (10% FCS). At 24h there are moderate architectural changes (sinusoidal expansion) with a degree of hepatocyte loss.

3.3.2.2. Cell viability assay

Morphology is a relatively crude method of assessing cell viability. To assess cell viability in a quantitative fashion a cell viability assay based on a commercial system was used (discussed in Chapter 2 Methods, section 2.2.1.2). A normalisation experiment was performed with different ratios of

viable and dead Huh 7.5 cells at different percentages (ratio of viable and dead cells; 100:0, 80:20, 60:40, 40:60, 20:80, 0:100 respectively). Dead cells were obtained by incubating them at 80°C for 10 minutes. Figure 3.9 shows effect on cell luminescence at different ratios of viable and dead cells.

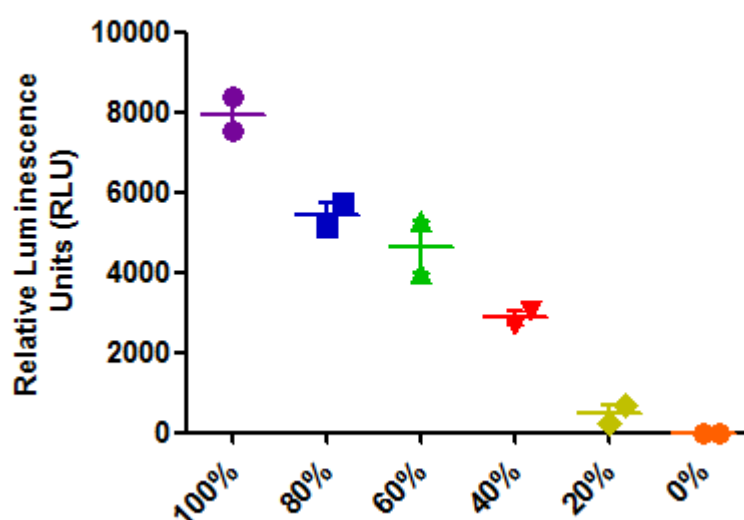


Figure 3.9: Normalisation of the cell viability assay: Viable and dead cells at different percentages starting from 100% viable cells (with 0% dead cells) to 0% viable cells (with 100% dead cells). Experiment was performed to see ATP luminescence (RLU) in different percentages of viable cells.

Cell viability was assessed in cells isolated directly from liver biopsy fragments after they were maintained in normal medium (DMEM + 10% FCS) for 24 and 48 hours. The results are shown in the figure 3.10 (1000 cells) and 3.11 (500 cells) which show the viability of intact liver biopsy fragments after incubating them for 24 and 48 hours and then isolating cells for assessments. In experiments where isolated primary human hepatocytes were incubated in

the medium for 24 and 48 hours and then assessed for viability are discussed in the section 3.3.4.2

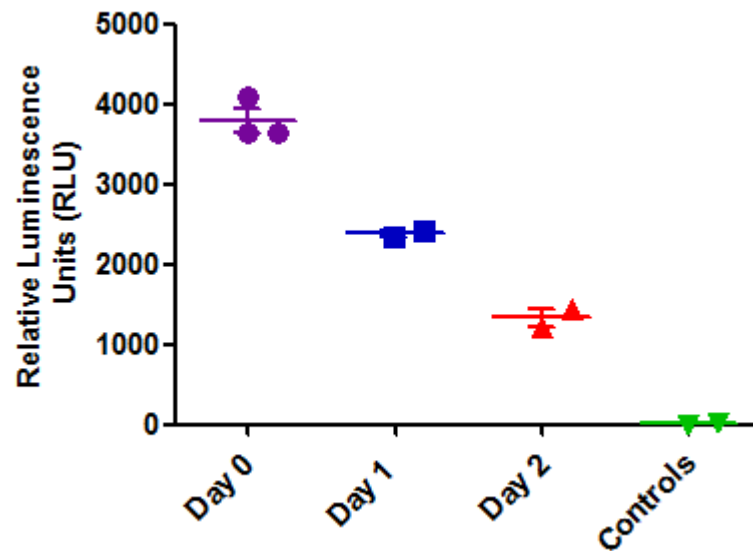


Figure 3.10: Cell viability after 24 and 48 hours in liver biopsy fragments incubated for 24 and 48 hours and is expressed as mean ATP luminescence (RLU). Total number of cells isolated from the intact fragment for assessment was 1000.

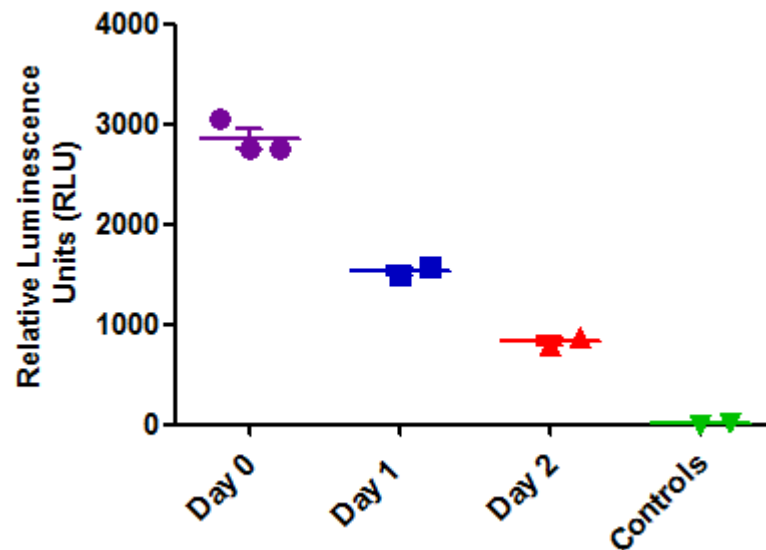


Figure 3.11: Cell viability after 24 and 48 hours in liver biopsy fragments incubated for 24 and 48 hours and is expressed as mean ATP luminescence (RLU). Total number of cells isolated from the intact fragment for assessment was 500.

In intact liver biopsies the mean luminescence fell to 62.8% (3626.5 RLUs to 2384.5 RLUs) after 24 hours and 35.2% (3626.5 RLUs to 1336.5 RLUs) after 48 hours when measured in 1000 cells after extraction. The mean luminescence fell to 53.8% (2860 RLUs to 1538.5 RLUs) after 24 hours and 29.3% (2860 RLUs to 838.75 RLUs) after 48 hours when measured in 500 cells after extraction. These data indicate that although cell viability is impaired after 24 hours a significant proportion of cells remain viable.

The same experiment was repeated after isolating the hepatocytes from the liver biopsy fragments and incubating these isolated primary human

hepatocytes for 24 and 48 hours in plates matted with type 1 collagen. These results are expressed later in this chapter in section 3.3.4.2.

3.3.2.3. Telaprevir concentration

Serum trough levels of telaprevir approximating 1.47nM are associated with marked inhibition of HCV replication in genotype 1 patients and *in vitro* studies show IC50 values for telaprevir ranging from 7nM (genotype 1) to 300 nM (genotype 3) (Perni, Almquist et al. 2006). Based on these data we chose a concentration of 14.7nM (10µg/ml) to evaluate the effects of telaprevir – i.e. concentrations in the non-trough range of those seen in patients treated with current dosing regimes. Though some data was obtained from a small number of samples, a full analysis of additional telaprevir concentrations was not possible due to limiting amounts of patient material. Lower concentrations of telaprevir (3.0nM) were tested on liver biopsies of four patients with genotype 1 HCV by Dr Linda Hibbert in a previous study in our laboratory. There was similar response at 3.0nM when compared to 14.7nM in one biopsy, two patients biopsies responded less well at a concentration of 3.0nM whereas one biopsy that did not respond at 14.7nM did not respond to 3.0nM either (data not shown). Attempts to use higher concentrations of telaprevir (37.0nM) were met with tissue death (identified from poor mRNA yields with low levels of β -actin) and this may reflect toxicity from the telaprevir solvent (100% DMSO).

3.3.2.4. NA808 concentration

Two different concentrations of NA808 (50nM and 500nM) were tested initially to examine the effects on HCV replication on three different chronic HCV patients (shown in figure 3.19). A concentration of 500nM was chosen for experiments because no significant conclusion could be drawn from using either 50nM or 500nM of NA808. This concentration was also used by 'Chugai Pharmaceuticals' in their prior experiments performed on replicon cells.

3.3.3. Experiments on liver biopsy fragments

3.3.3.1. Effect of telaprevir on liver biopsy fragments from chronic HCV patients

Given that liver biopsy fragments after 24 hours incubation have reasonable cell viability (arbitrarily defined as >50% survival) and maintain their morphology we addressed the question of whether they can be used to assess the efficacy of direct acting antiviral drugs. We chose the well studied protease inhibitor (telaprevir) at its effective concentration - 10µg/ml (14.7nM) and assessed its effects on HCV RNA expressions in biopsies of chronic HCV patients belonging to different genotypes. We reasoned that this rapidly acting antiviral agent should have activity in our assay and should reduce HCV replication, at least in patients with genotype 1 HCV. Liver biopsy fragments from thirty two patients belonging to major genotypes (1-3) were used to assess the effect of telaprevir after 24 hours. Patients' details are shown in the table 3.1.

No	Age	Genotype	Viral load	Fibrosis	Fold change
1	36	1	NA	2	-1.85
2	29	1b	6.04 log IU (1092400 IU)+	2	1.5
3	35	1b	6.69 log IU (4931750 IU)	3	-3.54
4	46	1b	NA	1	-4.88
5	53	1	5.57 log IU (372577 IU)	3	-9.26
6	26	1b	6.16 log IU (1446841 IU)	1	-1.29
7	31	1b	7.09 log IU (12332603 IU)	1	-1.19
8	41	1	NA	6	-1.02
9	48	1b	6.29 log IU (1946623 IU)	1	-137
10	49	1a/1b	6.64 log IU (4381228 IU)	3	1.027
11	56	1a	6.75 log IU (5671859 IU)	4	-1.1
12	55	1a	NA	1	-3.16
13	41	1	3.51 log IU (3237 IU)	4	-1.56
14	33	1a	6.8 log IU (6296858 IU)	3	2.4
15	30	1a	4.88 log IU (75457 IU)	1	-18.14

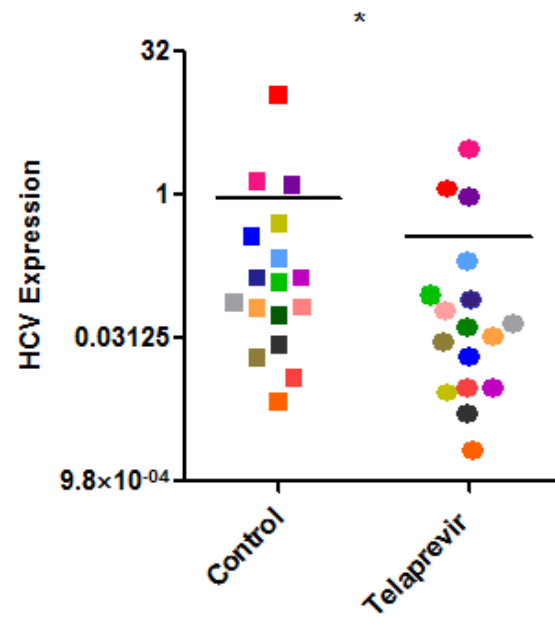
No	Age	Genotype	Viral load	Fibrosis	Fold Change
16	38	1b	5.39 log IU (245227 IU)	2	-16
17	64	1	6.19 log IU (1555694 IU)	1	-1.19
18	37	3	5.33 log IU (212095 IU)	3	1.68
19	43	3a	6.85 log IU (7034135 IU)	3	-1.39
20	53	3	6.91 log IU (8133832 IU)	3	-2.51
21	70	3	4.73 log IU (53093 IU)	1	1.83
22	39	3	6.89 log IU (7788994 IU)	1	1.79
23	45	3a	6.06 log IU (1155192 IU)	1	1.84
24	72	3	NA	2	4.95
25	25	3	4.87 log IU (74573 IU)	2	3.01
26	38	3a	6.21 log IU (1619920 IU)	3	6.33
27	34	3	4.68 log IU (47395 IU)	1	1.84
28	50	2	NA	1	1.84
29	43	2	5.99 log IU (978981 IU)	3	-3.04
30	27	2	4.86 log IU (71775 IU)	1	1

No	Age	Genotype	Viral load	Fibrosis	Fold Change
31	41	2	4.82 log IU (66109 IU)	1	-2.10
32	56	2	6.65 log IU (4417016 IU)	2	-1.30

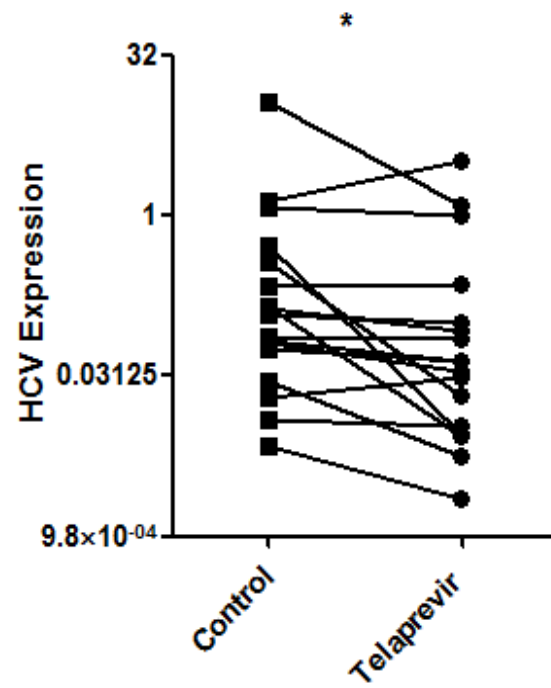
Table 3.1: Details of the patients whose liver biopsy samples were used in the experiments to look at HCV RNA gene expression after 24 hours of telaprevir treatment. The table shows age, genotype and subtype (if available), fibrosis score (Ishak scoring), viral load and fold change in HCV RNA after incubating these biopsy fragments in telaprevir compared to untreated samples from each patient. NA = no contemporary sample available for testing at the time of liver biopsy

Seventeen genotype 1 patients' biopsies were treated with telaprevir; untreated samples were taken as controls. Two control and two telaprevir treated samples were isolated for each patient and HCV RNA was quantified after real time qPCR. Figure 3.12 summarizes the results obtained after telaprevir treatment of biopsy fragments obtained from chronic HCV patients with genotype 1.

A)



B)



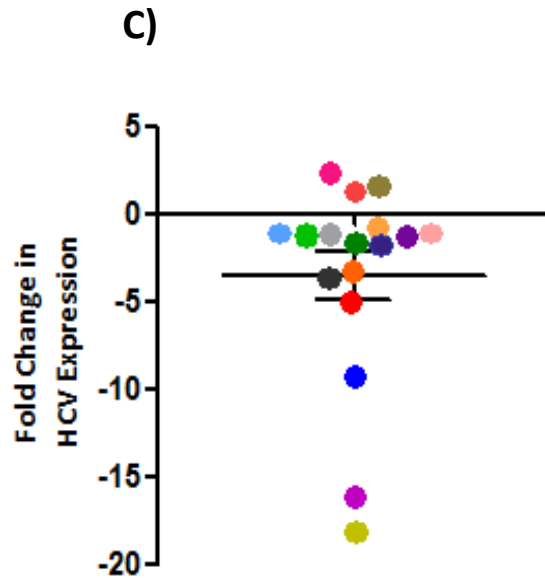
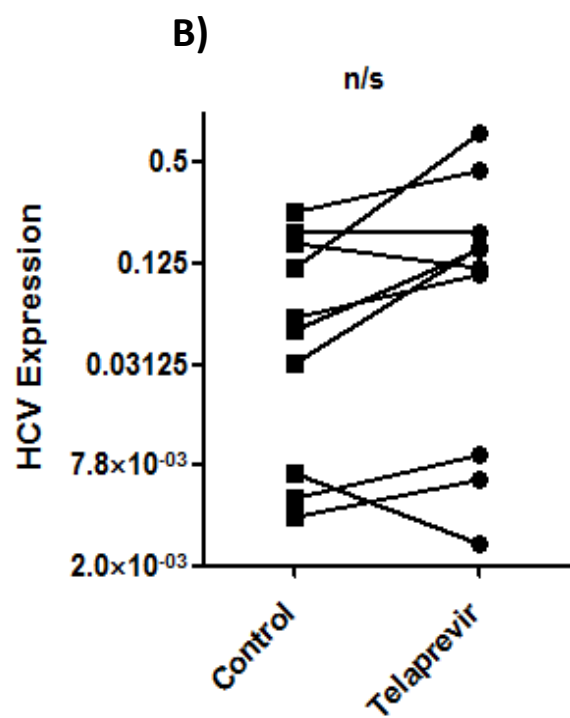
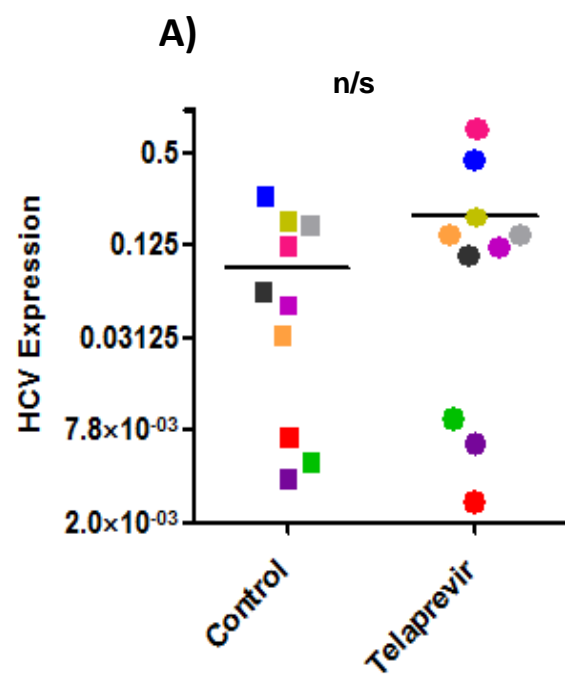


Figure 3.12: *Ex Vivo* drug testing using the liver biopsy fragments from patients with chronic HCV genotype 1 strain: A) The biopsy fragments were incubated with the medium alone or with telaprevir (10 μ g/ml) for 24 hours and the effects of telaprevir on HCV RNA expression was assessed by examining HCV RNA expression with real time quantitative PCR. (n=17, *p-value = 0.0138, using Wilcoxon matched pair test). Each point represents a mean of two samples taken from the same patient and is independent. B) The control and telaprevir treated samples are linked in this figure. C) Shows fold change in HCV expression in each patient's biopsy after treating with telaprevir. HCV expression represents the HCV RNA: β -actin ratio (calculated by the relative quantification method).

The mean viral RNA concentration in biopsies from patients with genotype 1 HCV (n=17) was significantly lower after 24 hours' incubation with telaprevir compared to unsupplemented medium (without telaprevir).

Ten genotype 3 patients' biopsies were treated with telaprevir, untreated samples were taken as controls. Two control and two telaprevir treated samples were isolated for each patient and HCV RNA was quantified after real time qPCR. Figure 3.13 summarizes the results obtained after telaprevir treatment of biopsy fragments from chronic HCV patients with genotype 3 strain.



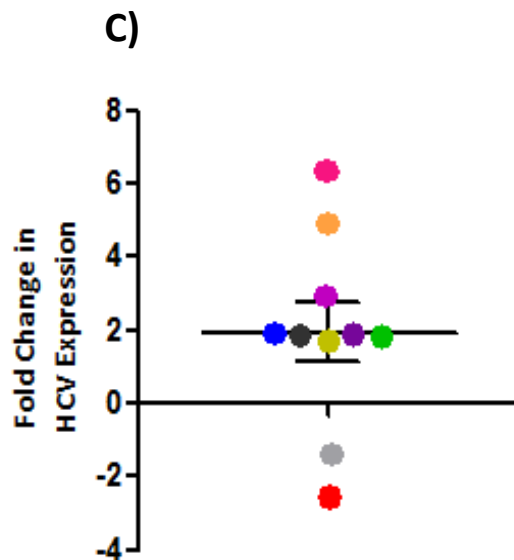
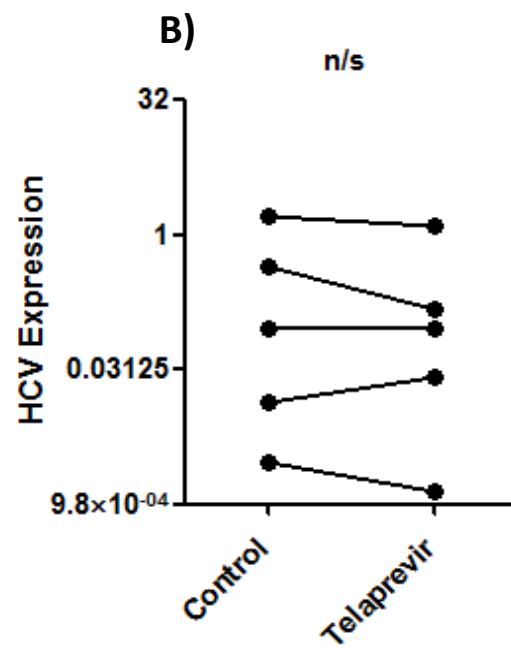
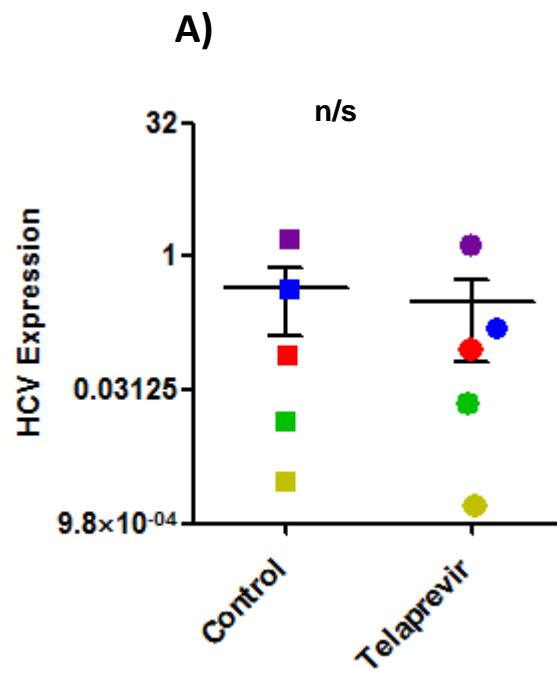


Figure 3.13: *Ex Vivo* drug testing using the liver biopsy fragments from patients with chronic HCV genotype 3 strain: A) The biopsy fragments were incubated with the medium alone or with telaprevir (10 μ g/ml) for 24 hours and the effects of telaprevir on HCV RNA expression was assessed by examining HCV RNA expression with real time quantitative PCR. (n=10, p-value >0.05, using Wilcoxon matched pair test). Each point represents a mean of two samples taken from the same patient and is independent. B) The control and telaprevir treated samples are linked in this figure. C) Shows fold change in HCV expression in each patient's biopsy after treating with telaprevir. HCV expression represents the HCV RNA: β -actin ratio (calculated by the relative quantification method).

Five genotype 2 patients' biopsies were treated with telaprevir and untreated samples were taken as controls. Two control and two telaprevir treated samples were isolated for each patient and HCV RNA was quantified after real time qPCR. Figure 3.14, summarizes the results obtained after telaprevir

treatment of biopsy fragments from chronic HCV patients with genotype 2 strain.



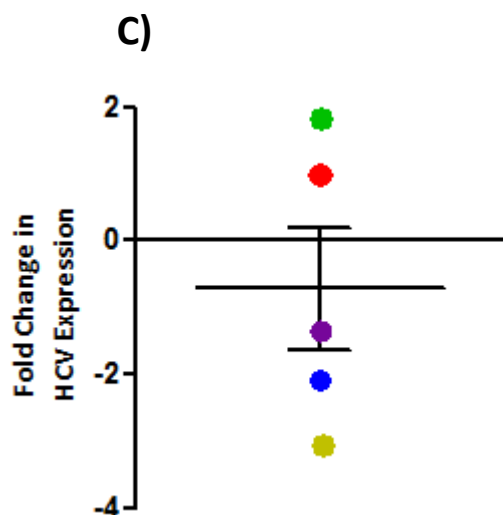


Figure 3.14: *Ex Vivo* drug testing using the liver biopsy fragments from patients with chronic HCV genotype 2 strain: A) The biopsy fragments were incubated with the medium alone or with telaprevir (10 μ g/ml) for 24 hours and the effects of telaprevir on HCV RNA expression was assessed by examining HCV RNA expression with real time quantitative PCR. (n=5, p-value >0.05, using Wilcoxon matched pair test). Each point represents a mean of two samples taken from the same patient and is independent. B) The control and telaprevir treated samples are linked in this figure. C) Shows fold change in HCV expression in each patient's biopsy after treating with telaprevir. HCV expression represents the HCV RNA: β -actin ratio (calculated by the relative quantification method)

These data show that telaprevir did not have any significant effect on HCV RNA levels in biopsies from patients with genotypes 2 or 3 HCV. Relatively few patients with genotype 2 strain of HCV were studied and therefore meaningful conclusions could not be drawn. For genotype 1 HCV there was a significant reduction in HCV RNA with telaprevir. However, the majority of biopsies showed a modest change in HCV RNA levels and the significant

effects of telaprevir may have been dominated by the very large changes seen in a small number of samples. For patients with genotype 3 HCV the changes with telaprevir were not statistically significant. Taken together these data suggest that telaprevir is more active against genotype 1 HCV compared to genotype 3. These data are compatible with data from clinical trials with telaprevir, suggesting that our liver biopsy model may be useful in examining the genotype sensitivity of different therapeutic agents. However, the changes seen in most liver biopsies were modest and large numbers of samples need to be analysed in order to examine the effects of the drug and to derive a statistically significant result. Furthermore, the need for multiple samples and statistical analysis detracts from the value of the assay – the data presented above indicate that it will be difficult to adapt this assay to determine the drug sensitivity of an individual patient. We speculate that the poor sensitivity of the assay may be related, at least in part, to the differences in HCV RNA expression in different fragments taken from the same biopsy. Data from initial co-experiments with Dr Linda Hibbert (shown below) indicated that there was a considerable sample to sample variation in untreated samples, suggesting that this may be a limiting factor with this approach. Figure 3.15 shows a summary of all experiments performed with liver biopsy fragments and includes data from 14 patients analysed by Dr Linda Hibbert merged with my experimental data.

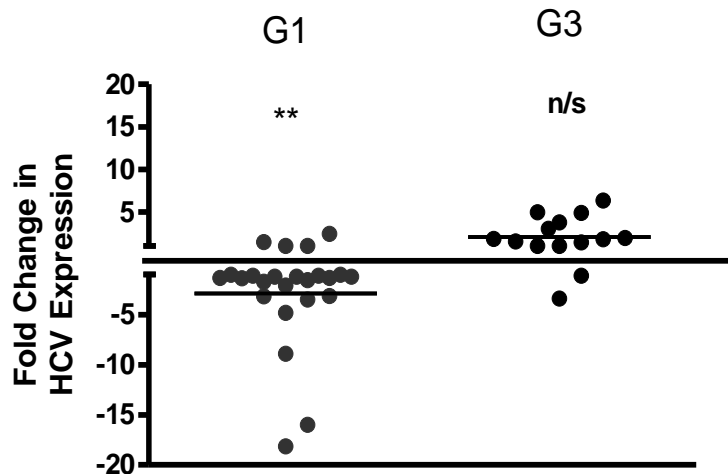


Figure 3.15: Effect of telaprevir on HCV expression in genotype 1 and 3:
The controls are samples from same liver biopsy fragments incubated in the absence of telaprevir. ****p=0.0054 (Wilcoxon matched pair test).**
Results are expressed as fold change. HCV expression represents the HCV RNA: β -actin ratio (calculated by the relative quantification method).

3.3.3.2. Effect of NA808 on liver biopsy fragments from chronic HCV patients

In order to examine the potential value of our biopsy fragment based approach we studied a second antiviral compound – NA808. NA808 is an experimental drug developed by Chugai Pharmaceutical Co. Ltd and is known to inhibit HCV replication in the genomic replicon system after 7 days incubation. It is a serine palmitoyl transferase inhibitor. Serine palmitoyltransferase (SPT) is an enzyme of the sphingolipid biosynthetic pathway. NA808 is thought to inhibit SPT activity because of its structural similarity to sphingosine which results in decreased intercellular

sphingomyelin and its intermediates. As sphingomyelin is one of the major components of the assembly of HCV this drug targets the assembly step of HCV (Lingwood, Kaiser et al. 2009, Simons and Ikonen 1997).

The two different concentrations of NA808 (50nM and 500nM) were tested initially to examine the effects on HCV replication on three different chronic HCV patients (illustrated in the figure 3.16)

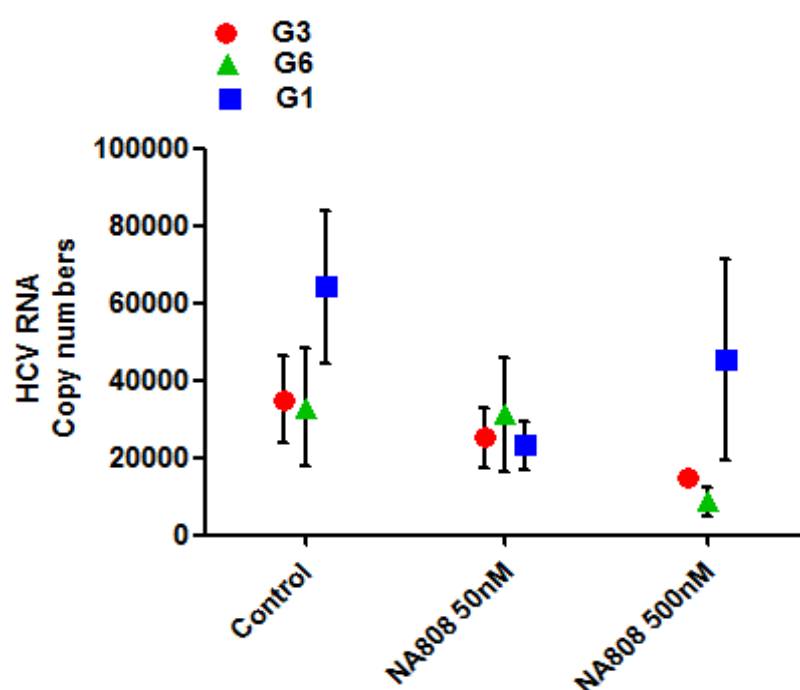


Figure 3.16: HCV RNA concentration in copy numbers evaluated by real time qPCR after using two different doses of NA808 (50nM and 500nM) and result obtained by absolute quantification method. (n=3). G = genotype.

Liver biopsy fragments from twenty five patients were used to assess the effect of NA808 after 24 and 48 hours. Details of these patients are discussed in tables 3.2 (patients whose liver biopsies were incubated with

NA808 for 24 hours) and 3.3 (patients whose liver biopsies were incubated with NA808 for 48 hours).

No	Age	Genotype	Viral load	Fibrosis	Fold Change
1	26	1b	6.16 log IU (1446841 IU)	1	6.50
2	31	1b	7.09 log IU (12332603 IU)	1	1.46
3	48	1b	6.28 log IU (1946623 IU)	1	1.40
4	41	1	NA	6	-1.08
5	49	1a/1b	6.64 log IU (4381228 IU)	3	-2.49
6	56	1a	6.75 log IU (5671859 IU)	4	-1.48
7	55	1a	NA	1	-1.16
8	41	1	3.51 log IU (3237 IU)	4	3.79
9	27	2	4.85 log IU (71775 IU)	1	1.06
10	48	2	4.62 log IU (42068 IU)	1	2.16
11	41	2	4.82 log IU (66109 IU)	1	1.02
12	45	3a	6.06 log IU (1155192 IU)	1	-5.19
13	72	3	NA	2	-6.02
14	25	3	4.87 log IU (74573 IU)	2	-1.22
15	32	4	3.76 log IU (5853 IU)	2	6.50

No	Age	Genotype	Viral load	Fibrosis	Fold Change
16	46	6a	5.89 log IU (788749 IU)	1	1.46

Table 3.2: Details of the patients whose liver biopsy samples were used in the experiments to look at HCV RNA gene expression after 24 hours of NA808 treatment. The table shows age, genotype and subtype (if available), fibrosis score (Ishak scoring) viral load and fold change in HCV RNA after incubating these biopsy fragments in NA808 compared to untreated samples from each patient. NA = no sample available for viral load testing at the time of liver biopsy.

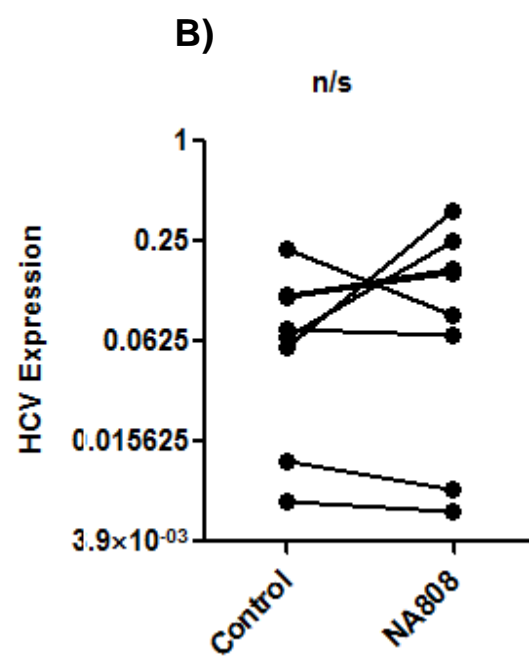
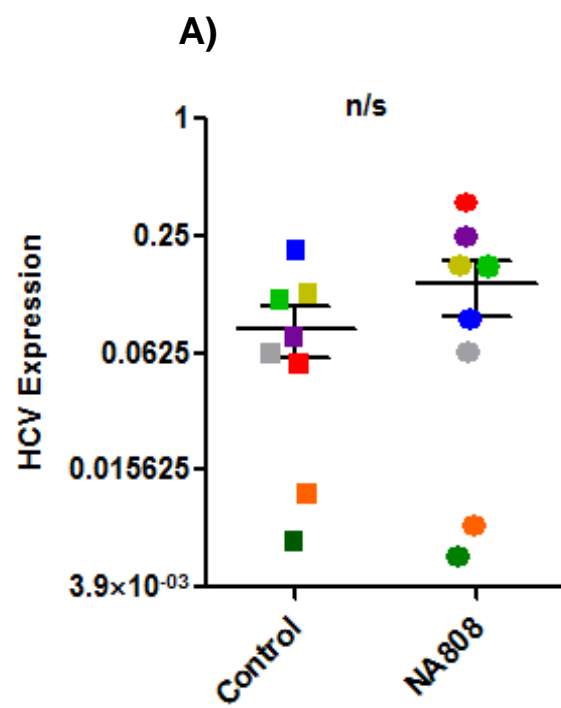
No	Age	Genotype	Viral load	Fibrosis	Fold Change
1	48	1	NA	3	1.17
2	33	1	NA	2	1.66
3	55	1	6.06 log IU (1164672 IU)	1	2.15
4	59	1	NA	3	177
5	33	1a	6.79 log IU (6296858 IU)	3	-6.94
6	30	1a	4.87 log IU (75457 IU)	1	11.55
7	38	1b	5.19 log IU (157792 IU)	2	-1.4
8	56	3a	6.60 log IU (3994255 IU)	4	-1.21
9	61	3a	6.62 log IU (4233360 IU)	4	-47.27
10	38	3a	6.2 log IU (1619920 IU)	3	-3.92
11	34	3	4.67 log IU (47395 IU)	1	-66.77

T

Table 3.3: Details of the patients whose liver biopsy samples were used in the experiments to look at HCV RNA gene expression after 48 hours of NA808 treatment. The table shows age, genotype and subtype (if available), fibrosis

score (Ishak scoring) and viral load from each patient. NA = no sample available for viral load testing at the time of liver biopsy

Figure 3.17, shows the effect of NA808 on genotype 1 strain of HCV after 24 hours incubations with the fragments of liver tissue (four from each patient-two controls and two NA808 treated).



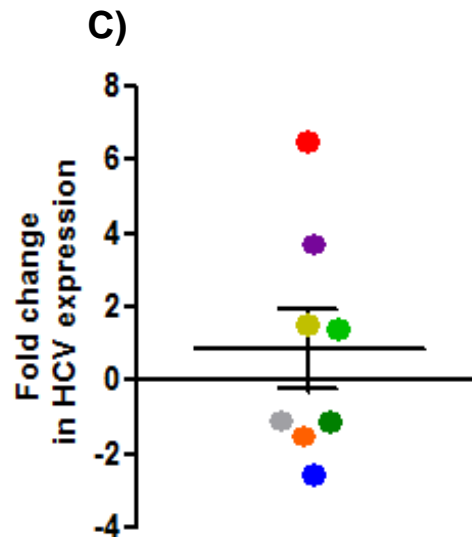
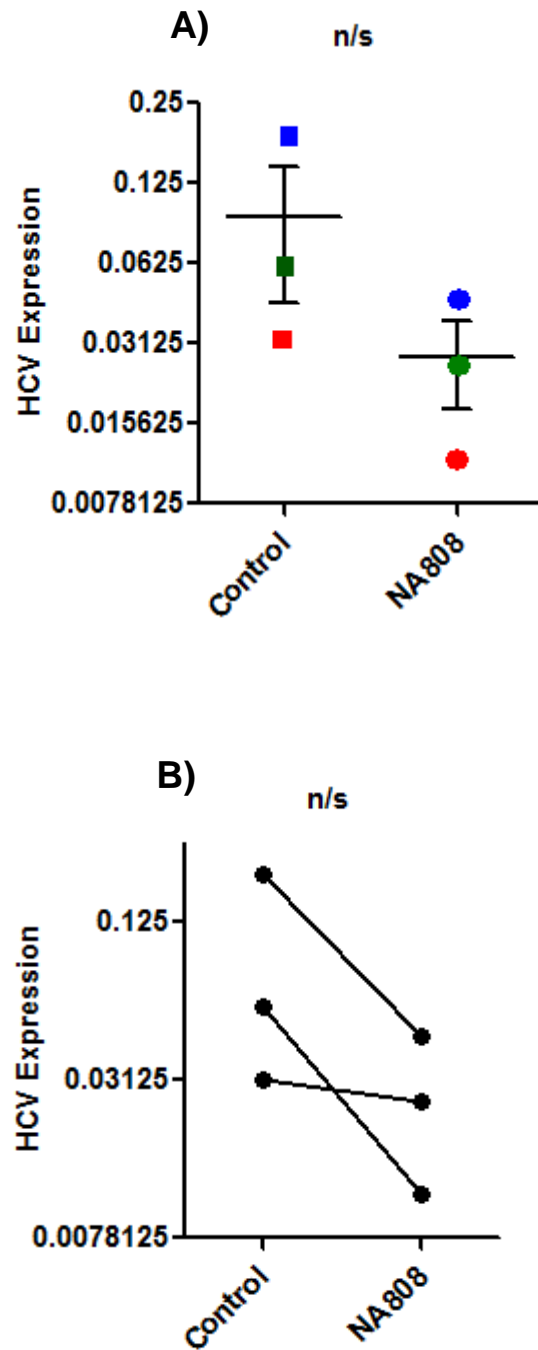


Figure 3.17: *Ex vivo* NA808 treatment of the infected liver tissues for 24 hours: A) This figure shows mean viral RNA concentrations after 24 hours incubation in NA808 compared to controls in genotype 1 strain infected liver biopsy fragments. Each point represents a mean of two samples taken from the same patient and is independent. B) The control and NA808 treated samples are linked in this figure. C) Shows fold change in HCV expression in each patient's biopsy after treating with NA808. HCV expression represents the HCV RNA: β -actin ratio (calculated by the relative quantification method)

Figure 3.18, shows the effect of NA808 on genotype 3 strain of HCV after 24 hours incubations with the fragments of liver tissue (four from each patient- two controls and two NA808 treated).



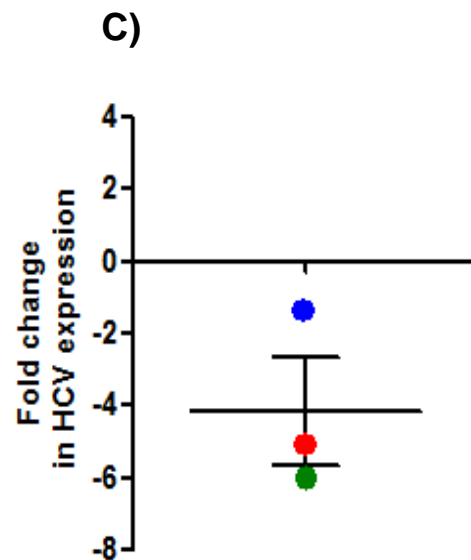
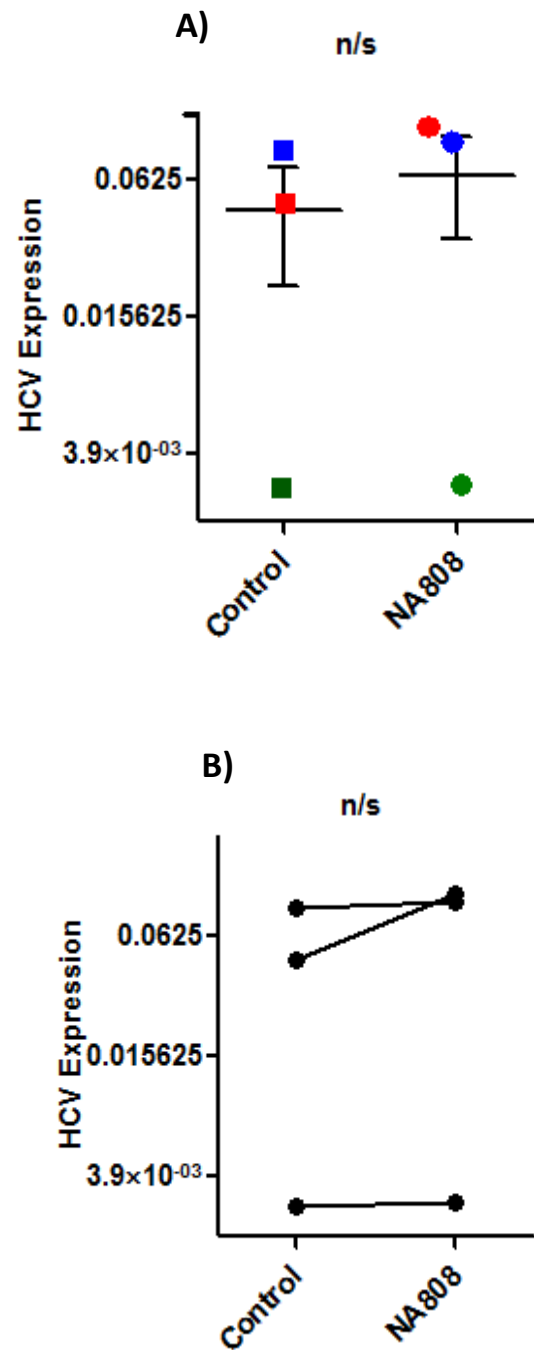


Figure 3.18: *Ex vivo* NA808 treatment of the infected liver tissues for 24 hours: A) This figure shows mean viral RNA concentrations after 24 hours incubation in NA808 compared to controls in genotype 3 strain infected liver biopsy fragments. Each point represents a mean of two samples taken from the same patient and is independent. B) The control and NA808 treated samples are linked in this figure. C) Shows fold change in HCV expression in each patient's biopsy after treating with NA808. HCV expression represents the HCV RNA: β -actin ratio (calculated by the relative quantification method)

Figure 3.19, shows the effect of NA808 on genotype 2 strain of HCV after 24 hours incubations with the fragments of liver tissue (four from each patient- two controls and two NA808 treated).



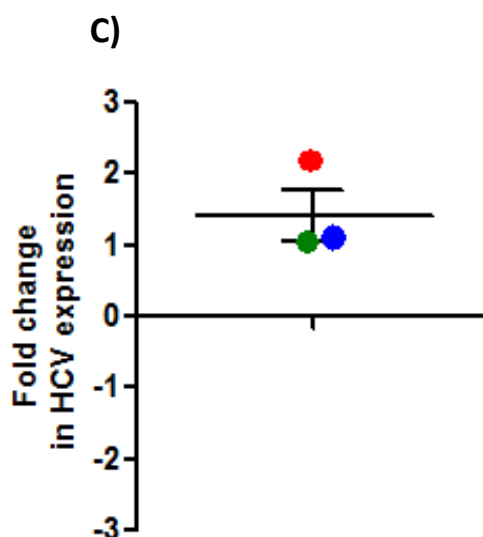
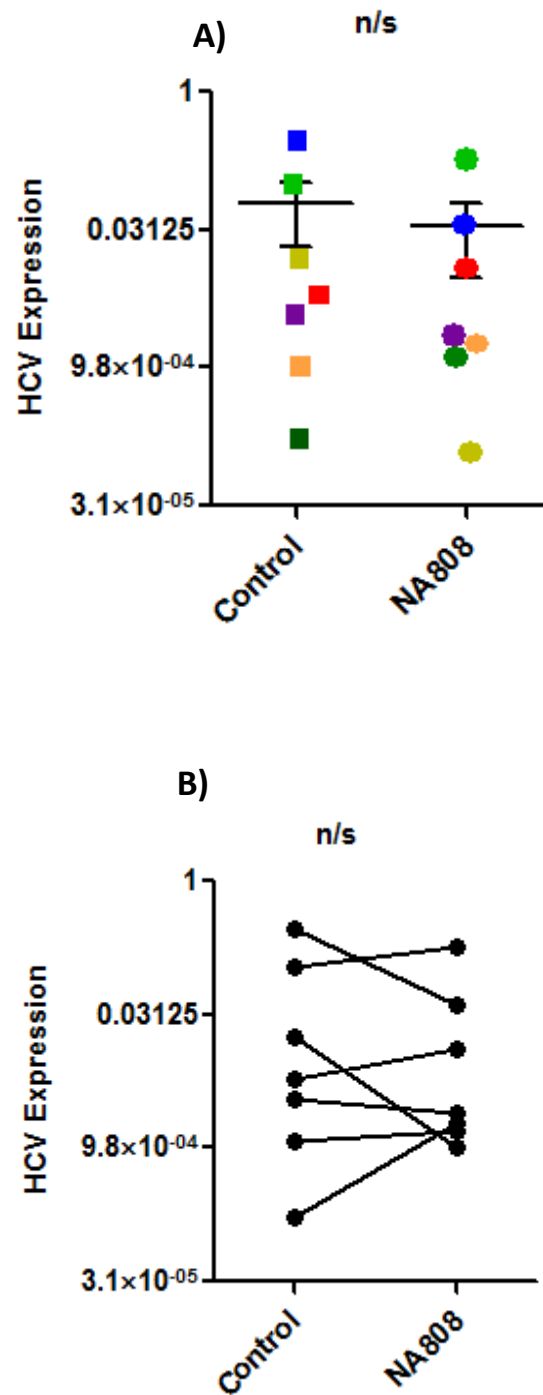


Figure 3.19: *Ex vivo* NA808 treatment of the infected liver tissues for 24 hours: A) This figure shows mean viral RNA concentrations after 24 hours incubation in NA808 compared to controls in genotype 2 strain infected liver biopsy fragments. Each point represents a mean of two samples taken from the same patient and is independent. B) The control and NA808 treated samples are linked in this figure. C) Shows fold change in HCV expression in each patient's biopsy after treating with NA808. HCV expression represents the HCV RNA: β -actin ratio (calculated by the relative quantification method).

This was a limited study with a small sample size and relatively short incubation period. Since in the genomic replicon system longer duration of exposure (>5 days) was required for NA808 to have an effect (unpublished data from Chughai pharmaceuticals), we tried extending the duration of incubation. To examine the effects of NA808 after 48 hours several liver biopsies were exposed to NA808 for 48 hours and HCV replication assessed further.

Figure 3.20, shows the effect of NA808 on genotype 1 strain of HCV after 48 hours incubations with the fragments of liver tissue (four from each patient- two controls and two NA808 treated).



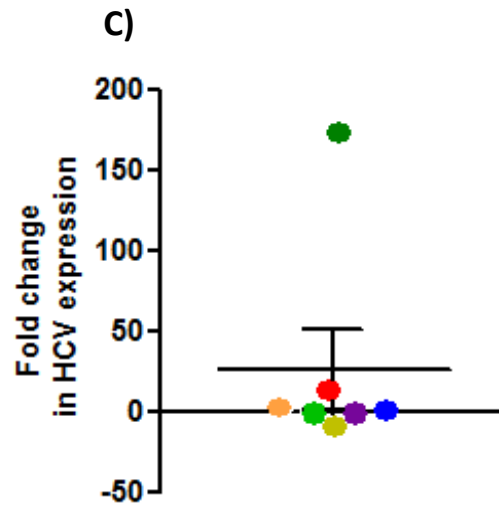
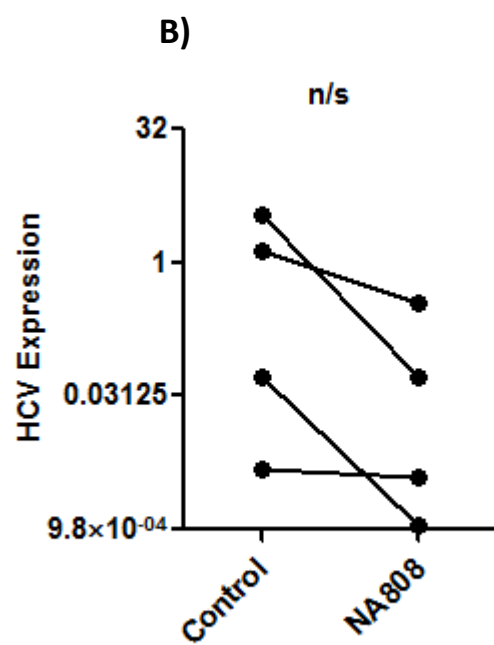
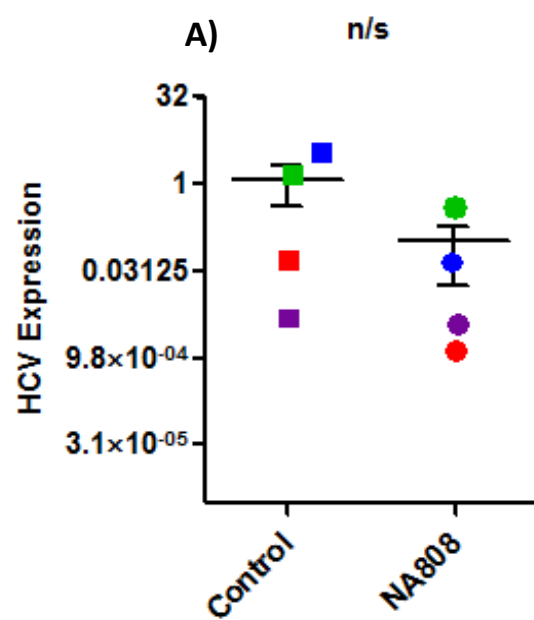


Figure 3.20: *Ex vivo* NA808 treatment of the infected liver tissues for 48 hours: A) This figure shows mean viral RNA concentrations after 48 hours incubation in NA808 compared to controls in genotype 1 strain infected liver biopsy fragments. Each point represents a mean of two samples taken from the same patient and is independent. B) The control and NA808 treated samples are linked in this figure. C) Shows fold change in HCV expression in each patient's biopsy after treating with NA808. HCV expression represents the HCV RNA: β -actin ratio (calculated by the relative quantification method).

Figure 3.21, shows the effect of NA808 on genotype 3 strain of HCV after 48 hours incubations with the fragments of liver tissue (four from each patient- two controls and two NA808 treated).



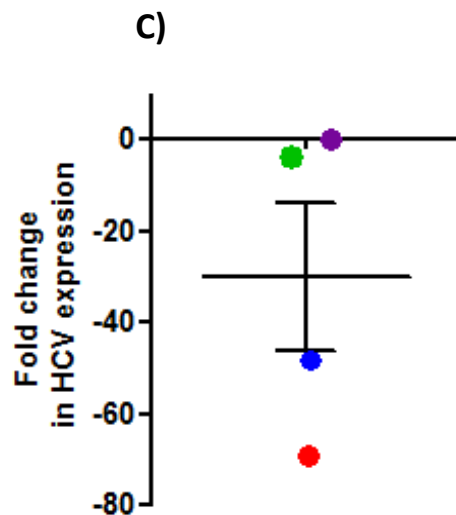


Figure 3.21: *Ex vivo* NA808 treatment of the infected liver tissues for 48 hours: A) This figure shows mean viral RNA concentrations after 48 hours incubation in NA808 compared to controls in genotype 3 strain infected liver biopsy fragments. Each point represents a mean of two samples taken from the same patient and is independent. B) The control and NA808 treated samples are linked in this figure. C) Shows fold change in HCV expression in each patient's biopsy after treating with NA808. HCV expression represents the HCV RNA: β -actin ratio (calculated by the relative quantification method).

Extending the duration of incubation to 48 hours gave us similar results to those seen after 24 hours. However, there are several limitations to this study – the tissue samples begin to die after 24 hours and tissue viability is markedly reduced; the sample size was very small and data from the replicon model suggests that NA808 may take several days to exert its antiviral effects. In next section we will discuss the measures we have taken to modify the assay to overcome these limitations.

3.3.4. Experiments on isolated hepatocytes

3.3.4.1. Isolated hepatocytes

In the above section we discussed drug testing on intact liver biopsy fragments. In this section we will discuss the work with hepatocytes isolated from the liver biopsy fragments. In intact liver biopsy fragments each fragment will contain a variable amount of liver tissue and stromal tissue (including fibrous tissue) and the proportion of infected cells will vary. We speculate that this fragment to fragment variability increased the sample to sample variability that adversely impacted our ability to identify drug induced effects. To overcome this limitation we speculated that isolated hepatocytes might allow homogenous cultures to be developed that can be used to assay drug activity. Here we describe our attempts to develop such a system.

3.3.4.2. Cell viability of isolated hepatocytes

After extraction from the liver biopsy, cell viability of isolated hepatocytes was assessed after 0, 24 and 48 hours using the ATP cell viability assay and cell viability was measured in relative luminescence units (RLU). Two different cell counts were used i.e.; 1000 and 500 cells. Figure 3.22 and figure 3.23 show the cell viability in 1000 and 500 cells at 0, 24 and 48 hours, respectively. Note that these experiments differ from the experiments outlined in above section when the viability of intact liver biopsy fragments was assessed.

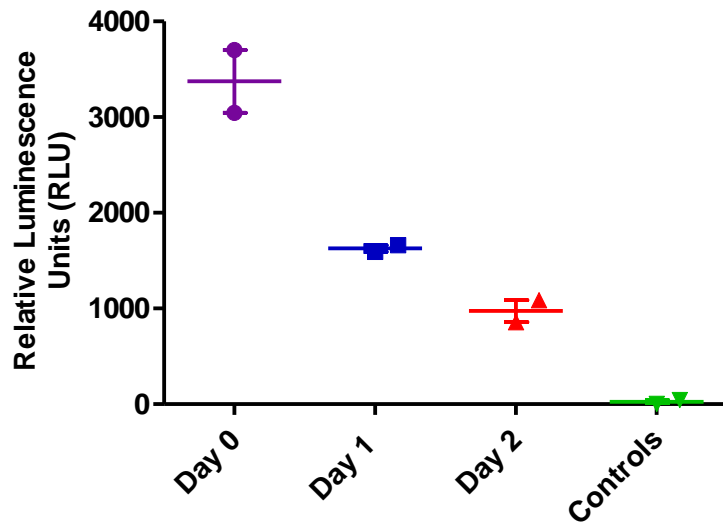


Figure 3.22: Cell viability after 24 and 48 hours in cells separated from liver biopsy fragments and incubated for the indicated times (using 1000 cells). The mean ATP luminescence is used to assess viability of these cells. Control is plain medium with no cells.

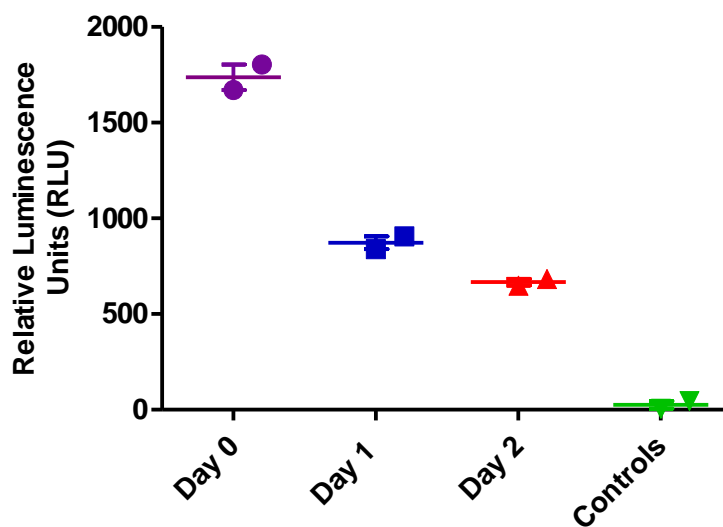


Figure 3.23: Cell viability after 24 and 48 hours in cells separated from liver biopsy fragments and incubated for the indicated times (using 500

cells). The mean ATP luminescence is used to assess viability of these cells. Control is plain medium with no cells.

In isolated cells the cell viability decreased to 44.8% (from 3626.5 RLU to 1626.8 RLU) after 24 hours and to 26.8% (3626.5 RLU to 973.8 RLU) after 48 hours in 1000 hepatocytes. In 500 hepatocytes cell viability came down to 50% (from 1737 RLU to 872.7 RLU) after 24 hours and to 38% (1737 RLU to 666.75 RLU) after 48 hours indicating that maintaining the integrity of the sample may improve cell viability while isolating the cells from their extracellular matrix causes them to die more quickly. Note that these pilot experiments were not performed in parallel and direct comparison of the data from these experiments and the experiments described in above section is not possible.

Nevertheless the data shown above indicates that isolated human hepatocytes have poor viability under standard culture conditions and indicates that examining the impact of drugs on isolated hepatocytes is unlikely to be productive. In these experiments we did not assess hepatocyte specific cell markers but others have shown that expression of hepatocyte specific markers is markedly reduced with simple culture of hepatocytes (Bhatia, Balis et al. 1999, Khetani and Bhatia 2008).

Table 3.4 shows the details of all the patients whose liver biopsy samples were tested for cells viability. This includes both those which were kept intact in culture and those isolated and then incubated for 24 and 48 hours.

No	Age	Genotype	Viral load	Fibrosis
1	53	1	5.13 log IU (134139 IU)	3
2	45	1	5.73 log IU (546020 IU)	4
3	77	1b	6.19 log IU (1573715 IU)	4
4	52	3a	6.4 log IU (2533182 IU)	2
5	49	3	5.32 log IU (208503 IU)	1

Table 3.4: Details of the patients whose liver biopsy samples were used in the experiments to look at cell viability at 24 and 48 hours. The table shows age, genotype and subtype (if available), fibrosis score (Ishak scoring) and viral load from each patient.

3.3.4.3. Pure hepatocyte culture versus co-culture

There are multiple assays to assess hepatocyte function including simple viability assays (used in our preliminary experiments), morphology, expression and activity of hepatocyte specific proteins, enzymes and soluble factors including albumin expression, urea production, cytochrome P450, phase II enzymes and glutamine synthetase activity (Bhatia, Balis et al. 1999, Khetani and Bhatia 2008). Since our aim was to develop a culture system to facilitate analysis of HCV replication we chose to monitor viability/differentiation by examining HCV RNA replication. The aim was that once an approach allowing HCV replication to be maintained for reasonable

periods of time was developed we planned to monitor hepatocyte status further using some of the assays outlined above.

A) HCV RNA expression in pure hepatocytes compared to co-cultures of hepatocytes and 3T3 embryonic fibroblasts (24 and 48 hours)

As noted above others have shown that the co-culture of hepatocytes with 3T3 murine embryonic fibroblasts grown in micro-patterns allows very prolonged culture of differentiated hepatocytes that can be infected with HCV. However, the micro-patterning technique is technically complex, allows only tiny clusters of hepatocytes to be grown and permits very limited replication of HCV. We suspect that such a complex procedure with the potential for significant sample to sample variation will prove difficult to develop for studies of individual drug sensitivity. Furthermore the micro-patterning technique is optimized to allow hepatocyte differentiation over many weeks. We speculate that if a simple, easy to use hepatocyte culture technique could be developed that would maintain hepatocytes in a sufficiently differentiated state to support HCV replication for a period of 48-72 hours then assessment of the effects of different antiviral agents would be possible. We therefore attempted to develop an 'adequate', rather than a 'perfect' hepatocyte culture.

Liver biopsies from ten patients with HCV were used to analyse the effects of co-culture with fibroblasts on HCV expression after 24 and 48 hours. Around 2×10^5 to 4×10^5 hepatocytes were separated from each biopsy and then

plated in 4 wells (collagen type 1 coated to provide an extracellular matrix component to hepatocytes). Two wells contained pure culture with only primary human hepatocytes (PHHs) and the other two wells contained co-culture of PHHs and murine 3T3 embryonic fibroblasts at a ratio of 1:4 before incubation. All wells contained equal number of hepatocytes. After 24 and 48 hours incubation we compared expression of HCV RNA by real time qPCR (discussed in chapter 2). HCV RNA expression in pure PHHs culture was analysed against co-culture of fibroblast and hepatocytes from each liver biopsy sample from chronic HCV infected individuals. Table 3.5 and 3.6 show details of patients whose liver biopsy samples were used in the experiments to look at HCV RNA gene expression in co-cultured cells after a 24 and 48 hours respectively.

No	Age	Genotype	Viral load	Fibrosis	Fold Change
1	34	3	5.49 logIU(312241 IU)	1	2.40
2	45	3a	5.25 logIU (179052 IU)	1	4.33
3	48	2b	6.69 logIU (4855028 IU)	2	3.93
4	34	4	5.22 logIU (165896 IU)	3	5.87
5	49	2b	4.43 logIU (27202 IU)	2	16.8
6	57	1a	6.50 logIU (3189531 IU)	3	1.5
7	38	3a	6.55 logIU (3547273 IU)	2	-1.058

Table 3.5: Details of the patients whose liver biopsy samples were used in the experiments to look at HCV RNA gene expression in co-cultured cells after 24 hours. The table shows age, genotype and subtype (if available), fibrosis score (Ishak scoring), viral load (done closest to the time when liver biopsy was taken from each patient) and fold change in HCV RNA gene expression in co-cultured cells compared to pure primary human hepatocyte cultures in biopsies of different HCV positive individuals.

No	Age	Genotype	Viral load	Fibrosis	Fold Change
1	63	3a	NA	4	3.462
2	47	1	6.29 log IU (1929423 IU)	3	-5.40
3	43	3b	5.09 logIU (116418 IU)	5	3.09

Table 3.6: Details of the patients whose liver biopsy samples were used in the experiments to look at HCV RNA gene expression in co-cultured cells after 48 hours. The table shows age, genotype and subtype (if available), fibrosis score (Ishak scoring), viral load (done closest to the time when liver biopsy was taken from each patient) and fold change in HCV RNA gene expression in co-cultured cells compared to pure primary human hepatocyte cultures in biopsies of different HCV positive individuals. NA = no sample available for viral load testing at the time of liver biopsy

Figures 3.24 and 3.25 show HCV RNA gene expression in co-cultured 3T3 and PHHs cells verses pure PHHs (24 hours and 48 hours respectively).

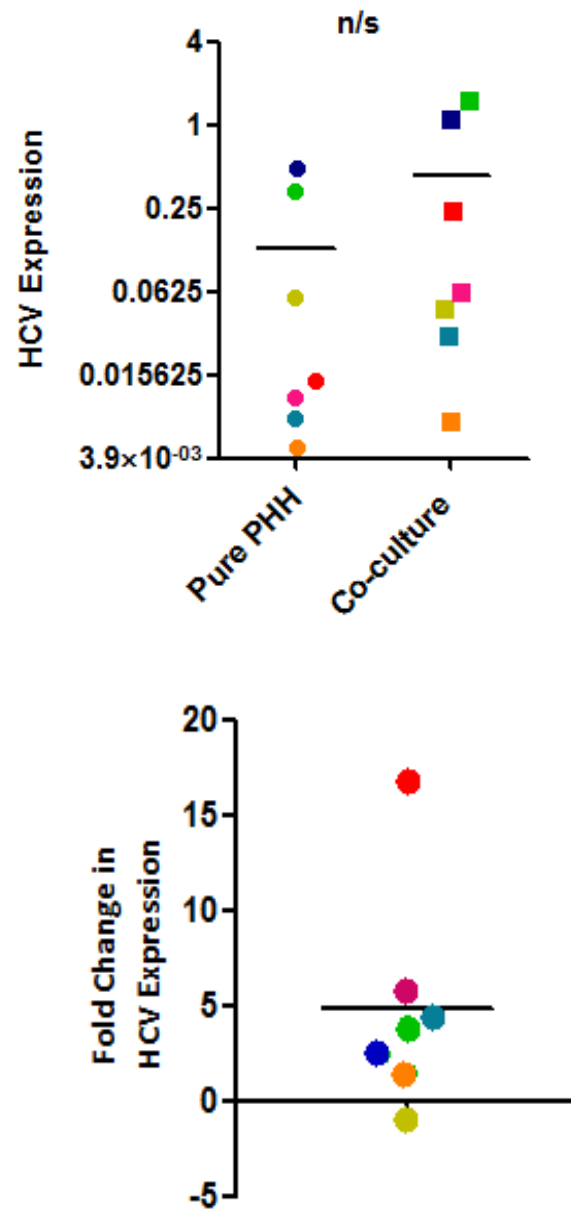


Figure 3.24 : Figure shows the HCV RNA concentration after 24 hours in pure primary human hepatocytes (PHHs) culture compared to that in the co-culture of PHHs and 3T3 fibroblasts (p-value = n/s, using Wilcoxon matched pair test). A) Shows the comparison of HCV expression among pure primary human hepatocytes cultures and co-cultures of fibroblast and hepatocytes. B) Shows the fold change in expression of HCV RNA after co-culturing relative to pure cultures.

Each point represents mean of duplicate wells from each pure- and co-culture. HCV expression represents the HCV RNA: β -actin ratio (calculated by the relative quantification method).

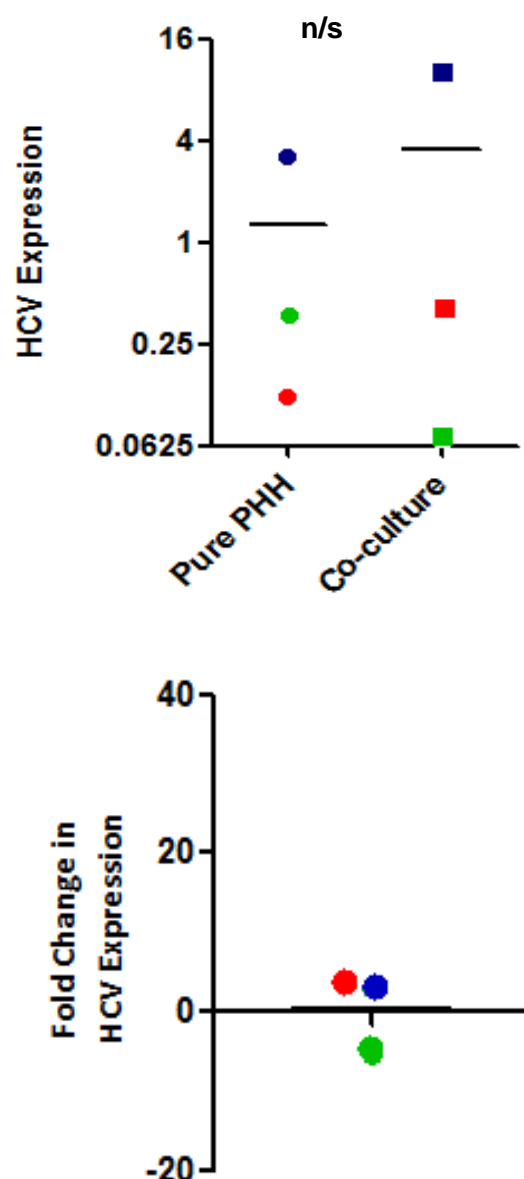


Figure 3.25: This figure shows the HCV RNA concentration after 48 hours in pure primary human hepatocytes (PHHs) culture compared to that in the co-culture of PHHs and 3T3 fibroblasts (p-value = n/s using Wilcoxon matched pair test). A) Shows the comparison of HCV expression among pure primary human hepatocytes cultures and co-

cultures of fibroblast and hepatocytes. B) Shows the change in expression of HCV RNA after co-culturing relative to pure cultures. Each point represents mean of duplicate wells from each pure- and co-culture. HCV expression represents the HCV RNA: β -actin ratio (calculated by the relative quantification method).

Although the small number (n=10) of samples studied did not allow a statistically significant difference in HCV expression to be detected between the two different culture approaches there was a clear trend towards increased HCV RNA expression in hepatocytes co-cultured with murine fibroblasts. The ease of this technique coupled with previous reports of the success of co-cultures (mentioned above) and the indication of an improvement in HCV RNA expression led us to try and refine this model further.

B) HCV RNA expression in co-cultures of PHH and 3T3 embryonic fibroblasts (48hours) using hepatocyte supplement medium

Liver biopsies from six patients with HCV were used to analyse the effect of hepatocyte supplement medium on co-culture of 3T3 fibroblasts and hepatocytes after 48 hours of incubation. Again around 2×10^5 to 4×10^5 hepatocytes were separated from each biopsy (as noted above) and then plated in 4 wells (collagen type 1 coated to provide extracellular matrix component to hepatocytes). Two wells contained co-culture of fibroblast with hepatocytes without hepatocyte supplement medium and the other two wells

contained co-cultures of primary human hepatocytes and murine 3T3 embryonic fibroblasts with hepatocyte supplement medium. After 48 hours incubation we compared expression of HCV RNA in the wells containing hepatocyte supplement medium with those containing none using real time qPCR (discussed in chapter 2 Methods). Table 3.7 shows details of patients whose liver biopsy samples were used in the experiments to look at HCV RNA gene expression in co-cultured cells with and without hepatocyte supplement medium.

No	Age	Genotype	Viral load	Fibrosis	Fold Change
1	59	3b	4.80 logIU (63324 IU)	3	5.25
2	43	3	NA	3	13.4
3	47	2	6.32 logIU (1700056 IU)	2	4.1
4	57	1	NA	3	6.27
5	43	1a	6.89 logIU (7754868 IU)	2	57.0
6	63	3a	6.45 logIU (2829228 IU)	2	1.13

Table 3.7: Details of the patients whose liver biopsy samples were used in the experiments to look at HCV RNA gene expression in co-cultured cells with and without hepatocyte supplement medium after 48 hours. The table shows age, genotype and subtype (if available), fibrosis score (Ishak scoring), viral load (done closest to the time when liver biopsy

was taken from each patient) and fold change in HCV RNA gene expression in co-cultured cells with and without hepatocyte supplement medium in different HCV positive individuals. NA = no sample available for viral load testing at the time of liver biopsy.

Figures 3.26, shows HCV RNA gene expression in co-cultured 3T3 and PHHs cells with and without hepatocyte supplement medium (incubated for 48 hours).

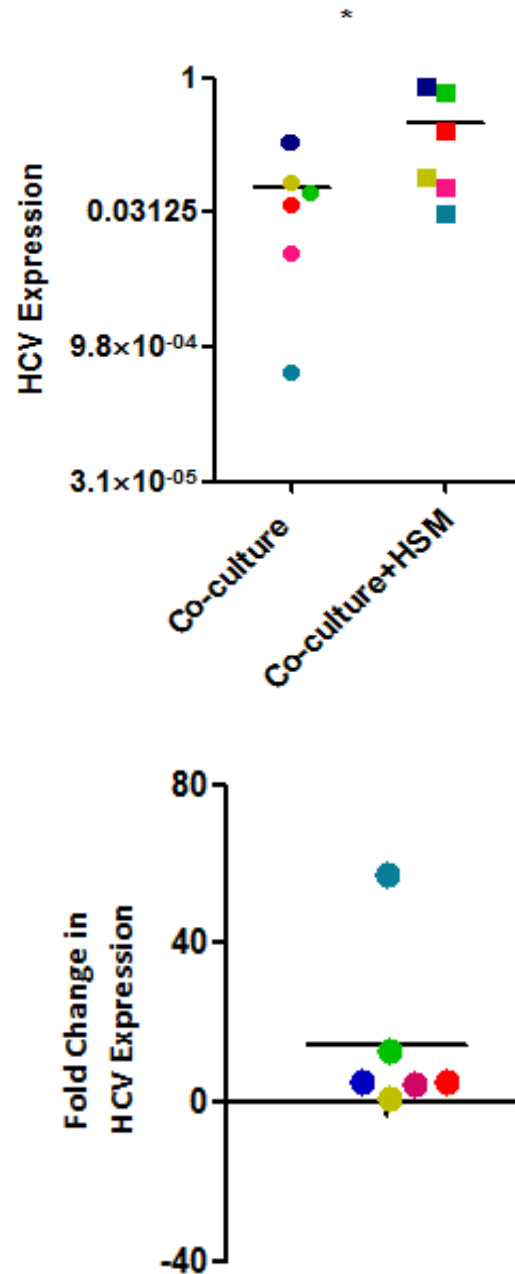


Figure 3.26: HCV RNA concentration after 48 hours in the co-culture of PHHs and 3T3 fibroblasts with and without hepatocyte supplement medium (HSM) (*p-value = 0.03 using Wilcoxon matched pair test). A) Shows the comparison of HCV expression among co-cultures of fibroblast and hepatocytes with and without hepatocyte supplement medium. B) Shows the change in expression of HCV RNA after co-culturing with and without HSM. Each point represents mean of

duplicate wells from each pure- and co-culture. HCV expression represents the HCV RNA: β -actin ratio (calculated by the relative quantification method).

Although the sample size studied in this experiment was small (n=6) there was a statistically significant increase in HCV RNA expression in co-cultures with hepatocyte supplement medium (HSM) compared to those without HSM (p=0.03). This may be because HSM supports the viability of hepatocytes and is required for their maintenance in cell culture or because it provides an optimized environment for hepatocytes and permits differentiation to a level that allows HCV replication to persist.

3.3.4.4. Drug validation experiment

A. Effect of telaprevir on HCV expression in co-culture model

Liver biopsies from seven patients (belonging to different genotypes) were used to isolate PHHs. These PHHs were co-cultured with 3T3 embryonic fibroblasts cells to produce a co-culture liver biopsy model for drug testing. Telaprevir was used at the concentrations discussed above - i.e.; 10 μ g/ml (14.7nM) and the effect on HCV RNA expression was observed after 48 hours of incubation with telaprevir. Table 3.8 shows details of patients whose liver biopsy samples were used in experiments to look at HCV RNA gene expression in co-cultured cells with and without telaprevir.

No	Age	Genotype	Viral load	Fibrosis	Fold Change
1	41	1	NA	3	1.85
2	48	2	6.66 logIU (4560203 IU)	3	1.24
3	48	1	6.34 logIU (2193653 IU)	3	-1.05
4	35	1	NA	0	4.5
5	43	3	6.64 logIU (4367581 IU)	1	1.55
6	80	2b	6.68logIU (4807015 IU)	2	1.77
7	57	1a	7.3 logIU (20000000 IU)	2	1.29

Table 3.8: Details of the patients whose liver biopsy samples were used in the experiments to look at HCV RNA gene expression in co-cultured cells treated with and without telaprevir after 48 hours. The table shows age, genotype and subtype (if available), fibrosis score (Ishak scoring), viral load (done closest to the time when liver biopsy was taken from each patient) and fold change in HCV RNA gene expression in co-cultures incubated with and without telaprevir from different HCV positive individuals. NA = no sample available for viral load testing at the time of liver biopsy.

The HCV expression (HCV/ β -actin ratio) in response to telaprevir treatment is illustrated in figure 3.27. For each biopsy obtained from a single patient four samples were taken; two controls and two experimental samples (telaprevir treated). Controls contained plain medium without telaprevir and were incubated for 48 hours before extraction.

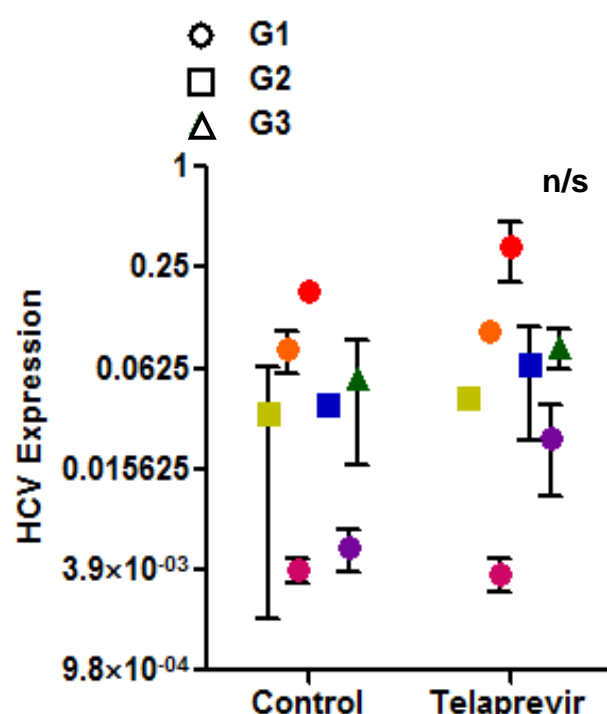


Figure 3.27: Effect of telaprevir on HCV RNA expression in the co-culture model containing different genotypes of HCV after 48 hours: Each point represents mean of two samples each of isolated PHHs treated and untreated (controls) with telaprevir, p-value = n/s and experiment shows high sample to sample variability (represented by standard error of mean). HCV expression represents the HCV RNA: β -actin ratio (calculated by the relative quantification method).

There was significant sample to sample variability observed between the samples of isolated human hepatocytes co-cultured with fibroblasts from each biopsy taken - two control and two experimental samples. It was observed that there was no significant difference in control and experimental samples (telaprevir treated) of co-culture model. This shows that attempts at extending the life of PHHs for 48 hours by co-culturing with fibroblast did not increase the likelihood of a better assessment of the effects of the antiviral agent telaprevir. This may be due to either co-culturing isolated PHH with 3T3 embryonic fibroblasts in a random manner doesn't enhance the ability of the cells to permit HCV replication or the distribution of HCV within liver cells is not homogeneous generating high levels of variability between different wells. Notwithstanding, the sample number was too small to derive any conclusion about the effect of telaprevir on different genotypes in an aggregated patient cohort. Likewise, it was decided to abandon this line of investigation due to above mentioned problems with this approach.

3.4. Discussion

Fragments of tissue from human liver biopsies can be maintained in tissue culture for up to 48 hours. However, the viability of the tissue is markedly reduced after incubation periods of greater than 24 hours. Data from intact liver biopsy fragments provides proof of the principal data showing that direct acting antiviral agents can be tested *ex vivo* using surplus liver biopsy material but it requires use of aggregated patient cohorts to observe the effect of telaprevir on different genotypes. Experiments with telaprevir reflect the findings of clinical studies indicating that telaprevir has efficacy in

genotype 1 but not genotype 3 HCV. Our data were unable to confirm reports showing that telaprevir is active against genotype 2 HCV as too few samples were available for testing. Although results described in this chapter indicate the viability of this approach there are significant problems with the assay as it now stands, in particular the large fragment to fragment variation and the heterogeneity of the response preclude an assessment of the individual response to telaprevir, significantly reducing the value of this approach.

To extend work with telaprevir a second antiviral agent was examined i.e; NA808. This is an experimental drug whose efficacy in patients is still under evaluation. However, data from the manufacturer show that the drug is a potent antiviral agent against HCV in replicon cells although prolonged incubation with the drug is required before antiviral activity is detected (suggested by data from Chughai Pharmaceuticals). In our studies, a considerable decline of HCV RNA was observed in biopsies infected with genotype 3 HCV after incubation with NA808 for 24 or 48 hours. However, we detected minimal response in biopsy samples from patients with genotype 1 HCV. It remains unclear whether the absence of an effect in patients with genotype 1 HCV is due to a slower onset of action of NA808 in this genotype or due to an absence of effect on this viral strain. To reach a firm conclusion regarding the significance of the effect of NA808 on genotype 3 viral strains increasing the sample size and extending the duration of incubation would have provided us with more accurate estimation of the effect of this drug. Although, reduced sample size is an issue here, it would be worth noting that NA808 affects lipid metabolism by inhibiting the sphingolipid biosynthesis.

HCV genotype 3 is associated with unique interactions with host lipid metabolism. There are multiple mechanisms by which HCV genotype 3 up-regulates lipogenesis and steatosis. It activates a mediator of steatosis called sterol regulatory element-binding protein in its host (Kim, Hong et al. 2007). Moreover, HCV genotype 3 core proteins down-regulate components which suppress lipid droplet formation (Clement, Peyrou et al. 2011). Steatosis has also been linked to higher levels of viremia in those infected with HCV genotype 3 strains and successful eradication of virus led to reduction in liver steatosis (Kumar, Farrell et al. 2002, Lonardo, Loria et al. 2006). Additionally, in HCV genotype 3 infections, steatosis is linked with increased viral relapse (Shah, Patel et al. 2011). NA808 can be studied further for its link with lipid metabolism and effect on different HCV genotypes.

Because of the inability to keep considerable numbers of PHHs viable beyond 24 hours and toxicity in man at concentrations which were effective *in vitro*, we did not study NA808 further. Moreover, experiments with intact fragments of human liver biopsies indicate that the tissue is viable for no longer than 48 hours and that there is considerable intra-assay variation, leading to a requirement for multiple analyses to derive significant results. This detracts us from the main aim which was to evaluate drug sensitivity in individual patient.

The results obtained from co-cultured hepatocytes replicate the studies performed by others suggesting that murine embryonic 3T3 cells facilitate the culture of human hepatocytes and suggest that hepatocytes show conservation of their functions and express soluble and insoluble proteins and enzymes in a more functionally stable and structurally conserved micro-

environment (discussed in Introduction 3.1). Although the sample number used in our comparative study of co-culture versus pure culture was small (n=10) and though results did not show significance regarding the rise in HCV expression there was an indication that HCV RNA expression improved in co-cultured samples and there was significant enhancement of HCV expressions in co-cultures treated with hepatocyte supplement medium.

Taken together these data persuaded us that further studies in this area were worthwhile and characterizing the differentiation of hepatocytes using expression of liver specific markers as well as examining the effects of antiviral agents on HCV replication would be a useful future intervention. We speculated that co-culture techniques coupled with using collagen coated plates and special medium (HSM) to keep hepatocyte metabolically active, will provide an easy to use model for drug testing in HCV infected patients. However, our experiments with telaprevir did not prove to be a model which could be replicated for drug testing. This model showed significant sample to sample variability between two samples plated out in duplicates as controls (untreated with telaprevir) and telaprevir treated. Moreover, work to prolong the viability of primary hepatocytes in culture did not provide acceptable results because of even wider sample to sample variation in PHHs co-cultured with fibroblasts without using the micro-patterning technique. It was not possible to perform micro-patterning and as discussed earlier micro-pattern co-culture experiments have not been possible outside the originators laboratory because of the unavailability of the stensil used in these studies (discussed in introduction section of this chapter).

Chapter Four

HCV Fusion Model

4.1. Introduction

4.1.1. Polyethylene glycol mediated forced cell-to-cell fusion

Artificial forced cell-to-cell fusion is used in various biotechnical research experiments and it can be carried out *in vitro* by using different methods which involve chemical, electrical and laser induced fusion. Chemical fusion by polyethylene glycol (PEG) was used as a method to induce cell-to-cell fusion in experiments performed in this thesis. PEG is a highly hydrated compound that brings cellular membranes to near molecular contact, making water between them thermodynamically unfavourable. This disrupted repacking of contacting layers of cellular membranes is necessary to induce fusion. It starts with the mixing of outer leaflet lipids, forming transient intermediate containing pores which join to form fusing compartments. The first intermediate transforms in a second intermediate in 1-3 minutes which then 'pops' to form a proper fusion pore with inner leaflet mixing followed by cell contents mixing. These sequence of events and activation energies correlate well to the process of cell fusion (Lentz and Lee 1999).

Cellular fusion is a common physiological process (discussed in chapter 1 section 1.10). We used artificial fusion in an attempt to transfer HCV from monocytes to replication competent hepatoma cells. In this chapter I describe my studies on PEG 1500 forced fusion with monocytes from HCV patients and a hepatoma cell line in an effort to create a model for the understanding of HCV replication.

4.1.2. Cell-based fusion assay for *in vitro* studies of HCV

As discussed in Chapter 1 Introduction, section 1.9, HCV is known to infect monocytes (Laskus, Radkowski et al. 2004) and HCV transfer from B cells to hepatocytes is established (Ducoulombier, Roque-Afonso et al. 2004). In one study umbilical cord derived macrophages were infected with HCV from patients' plasma or serum and the supernatant derived from HCV infected macrophage culture was used to infect other cell lines like B and T cell, lymphoid and neuronal cells. As hepatocytes infection was not tried by this method it is not known whether infection of hepatocytes would take place using this approach (Revie, Alberti et al. 2010). Moreover, HCV from patients' serum has not been propagated in hepatocytes in previous studies (Gondeau, Pichard-Garcia et al. 2009). The reasons for the failure of patient derived HCV to propagate and replicate in cultured human hepatocytes are not known but work in this thesis speculates that poor entry (perhaps because of poor expression of receptors) may play a role and rectifying this hurdle might overcome this issue. Thus, transfer of monocyte incorporated HCV might be possible by avoiding the HCV entry step. Moreover, it is proposed that if large amounts of virus can be delivered through this may overcome host inhibitors of replication. These speculations raised the question of whether fusion of HCV containing monocytes to hepatocytes might allow HCV propagation and replication to become established and this will be demonstrated in experiments explained in this chapter.

Polyethylene glycol was used to induce forced fusion of CD14 bead separated monocytes obtained from chronic HCV patients (with active viral

infection) and Huh 7.5 cell line (details explained in Chapter 2 Materials and Methods section 2.2.2) in an effort to make a hybrid cell line which contains genetic material from both CD14+ monocytes and Huh 7.5 cells to produce an *in vitro* model of hepatocyte infection which was grown for varying lengths of time prior to real time qPCR measurement of HCV RNA. This approach was also used to consolidate the knowledge obtained from previous studies which demonstrated establishment of viable HCV in monocytes and macrophages of chronic HCV patients. Polymerase chain reaction and immunofluorescence microscopy (both confocal and non-confocal) were carried out primarily to determine the presence and replication of HCV in the fused cells or their progeny.

4.2. Aims and hypothesis

The aim of this chapter is to establish a cell-based assay for the *in vitro* study of HCV infection and to determine whether fusing infected monocytes from patients with chronic HCV to replication 'permissive' liver cell line will lead to generation of hybrid cells harbouring viable HCV. We speculate that replication can be enhanced in hepatocyte/monocyte fusion model by creating an immune deficit environment and that monocytes can be used to capture HCV from sera of HCV patients and infect Huh 7.5 cells after fusion.

4.3. Results

4.3.1. Insight into initial fusion experiments

Initially, Huh 7.5 cells and monocytes were fused under different conditions to obtain the most favourable settings that would achieve maximum fusion between monocytes and Huh 7.5 cells. It was speculated that this optimization would achieve higher levels of fusion between cells consequently more monocytes and Huh 7.5 cells would fuse maximally to form a putative, hybrid (fused) cell line with minimal amounts of single cells in culture. This higher degree of fusion would provide more chance of HCV being transferred to the replication permissive liver cell line and result into establishment of HCV infection by circumventing HCV entry step.

Different techniques were tried to achieve optimal fusion i.e.; by fusing cells at different ratios i.e.; 1:2, 1:1 and 2:1 of monocytes and Huh 7.5 cells respectively, by using different incubation times with PEG 1500 ranging from one to seven minutes before addition of DMEM (with 10% FCS) for dilution and deactivation of PEG and by using PEG 1500 with different pHs to determine the ideal pH to gain highest level of fusion. Moreover, cells were examined for 24 hours and it was observed that the fused cells which were grown on non-collagen coated plates showed massive cell death in the initial days after fusion and empirical experiments with collagen coated plates facilitated cell survival. We also tried modifying the fusion reaction volume to keep cells closer together for first 24 hours which elucidated that using 500 µl DMEM (FCS 10%) in each well of a 12 well plate enabled cells to be in close

contact with each other and with the lower surface of the well and helped them to adhere and grow at the bottom of the well more effectively. It was contemplated that cell-to-cell interaction would play a crucial role in growth, development and proliferation of fused cell line. Hence, an optimal balance was eventually achieved which gave maximum fusion along with minimum cell membrane damage. Below are the details of these optimization experiments.

4.3.2. Determining optimal fusion conditions

A. Experimental controls

To generate control cells to monitor fusion we labelled monocytes and Huh 7.5 cells with different fluorescent dyes (fluorophores; DiI and DiO respectively). The techniques used to label the cells are described in Materials and Methods. To determine whether cells were appropriately labelled, DiI and DiO vibrant dye stained monocytes and Huh 7.5 cells were examined by flow cytometry. Labelled cells were then fused and investigated by FACS (fluorescence activated cell sorting) analysis. Figure 4.1 shows typical results from a flow cytometry analysis.

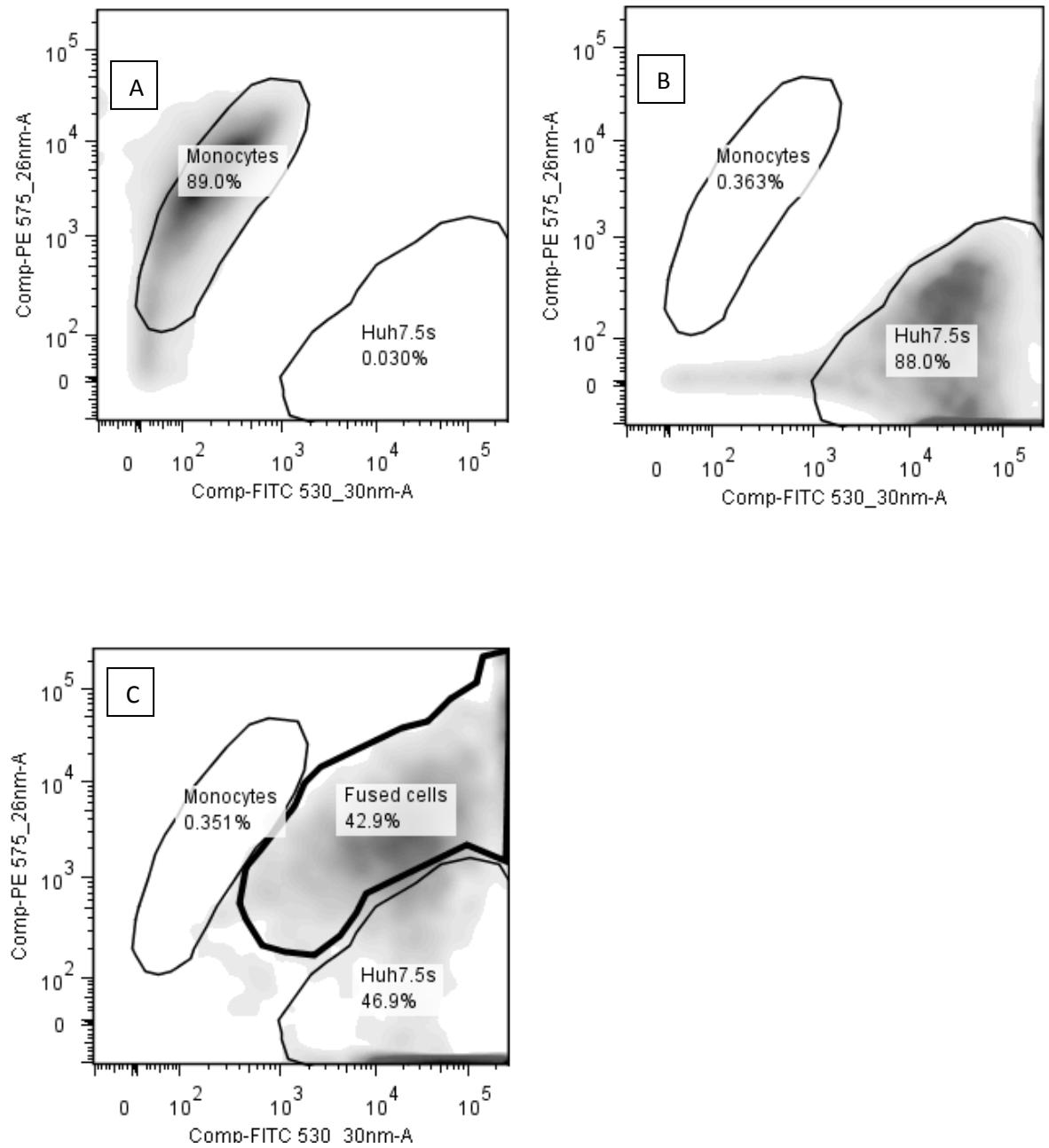


Figure 4.1: Controls for flow cytometric (FACS) analysis: A) Dil dye-labelled monocytes B) DiO dye-labelled Huh 7.5 cells C) Dil labelled monocytes and DiO labelled Huh 7.5 cells fused together by PEG 1500 at 1:1 ratio. Percentage of fused cells was assessed by flow cytometry (FACS) analysis.

B. Ratios of monocytes and Huh 7.5 cells for fusion

Dye labelled monocytes and Huh 7.5 cells were fused in different ratios i.e. 2:1, 1:1 and 1:2 and results analysed through flow cytometry experiments. The example shown below demonstrates extent of fusion depending on ratio of Huh 7.5 cells and monocytes. Percentage of fused monocytes and Huh 7.5s was 52.1% when fused in 1:1 ratio (unfused Dil labelled monocytes were 3.33% and DiO labelled Huh 7.5 cells were 0.14%). When fused at 2:1 monocyte to Huh 7.5 cell ratio percentage of fused monocytes and Huh 7.5s was 24.6% (unfused Dil labelled monocytes were 57% and DiO labelled Huh 7.5 cells were 4.82%) whereas percentage of fused monocytes and Huh 7.5s was 35.1% when fused in 1:2 monocyte to Huh 7.5 cell ratio (unfused Dil labelled monocytes were 2.96% and DiO labelled Huh 7.5 cells were 43.5%). Please see figure 4.2 for details. These experiments were performed on three separate occasions and although the absolute values differed the trend remained consistent and therefore a ratio of 1:1 was selected for subsequent experiments.

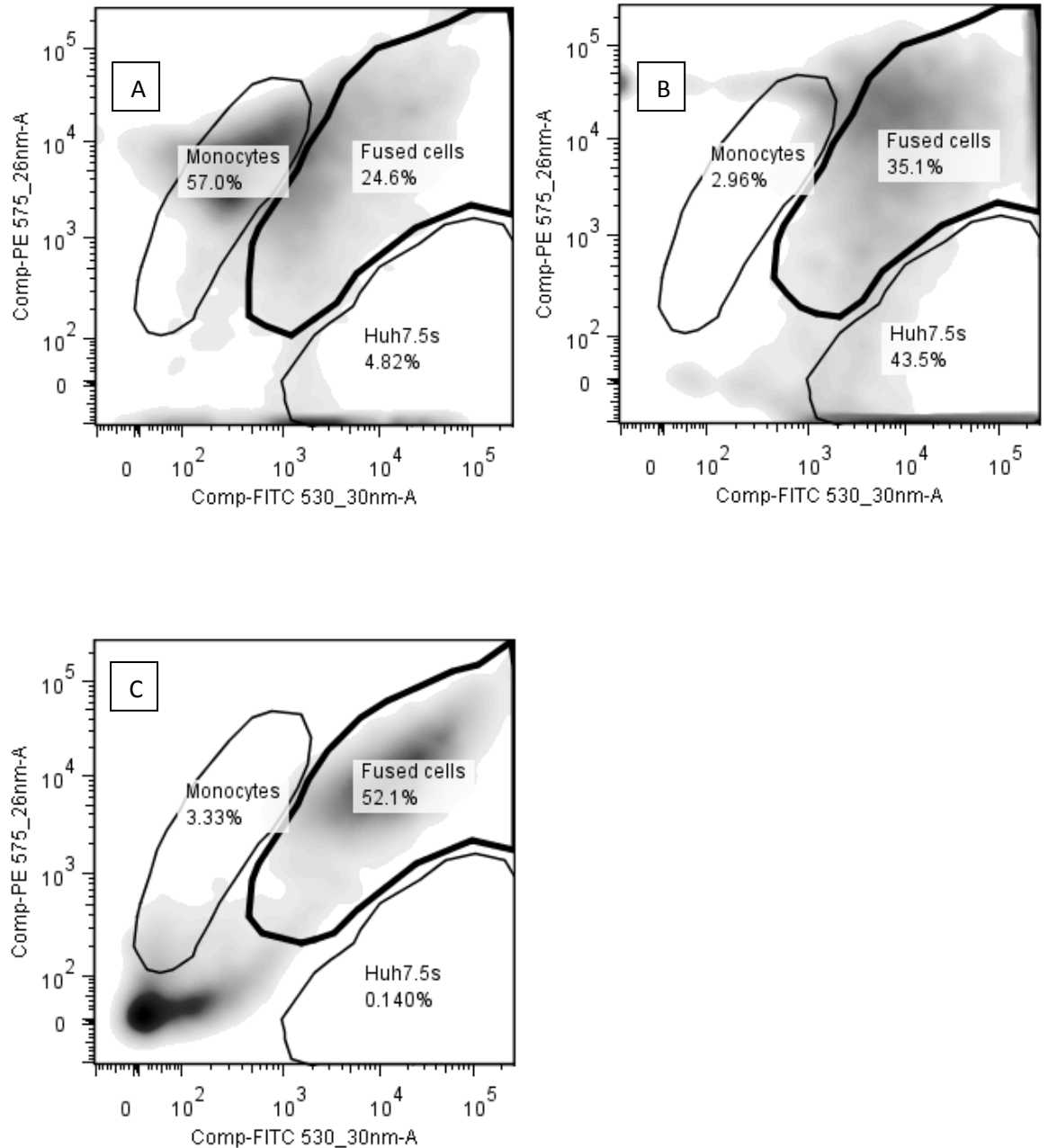


Figure 4.2: Huh 7.5 cells and monocytes fused at different ratios: Dye-labelled monocytes and Huh 7.5 cells incubated together in PEG 1500 at the ratio of 2:1 (A), 1:2 (B) and 1:1 (C), monocytes to Huh 7.5 cells and percentage of fused cells was assessed by flow cytometry (representative example from 3 separate experiments).

It was also observed that cell growth was better when fused at 1:1 monocyte to Huh 7.5 ratios where as at 2:1 and 1:2 monocyte to Huh 7.5 cell ratios cell

growth was noted to be poor by direct observation of the culture plate in three separate experiments.

C. Optimal cell number per well

Having identified 1:1 as the optimal fusion ratio we modified the number of cells in a fixed volume of reaction mixture. 0.5×10^6 fused cells were plated per well in collagen coated 12 well culture plates after PEG fusion within 500 μ l of medium for the first day. Using larger amounts of medium for the first day reduced adhesion with the bottom of the well, perhaps because of lack of close approximation to the base of the collagen coated well and reduced cell-to-cell interactions. These results were derived from gross observations under the microscope with greater number of floating (dead) cells (stained with trypan blue to check for viability) and lack of cellular confluence at the bottom of the well one day after fusion, based on 3 experiments performed without formal comparative quantification.

After 24 hours, when considerable cellular adhesion and growth was observed, medium was increased to 1ml in each well and old medium was replaced. This gave cells ample amount of time to recover from PEG induced membrane fragility. Since this plating density provided reasonable numbers of viable cells, experiments with different cell densities were not performed and this cell number was used in subsequent experiments.

D. PEG 1500 incubation time

To determine the optimal PEG incubation time DiO and Dil labelled Huh 7.5 cells and monocytes were kept in PEG 1500 at different durations and percentage of fusion was observed by flow cytometry (FACS analysis). The aim of optimising duration of incubation in PEG 1500 is to establish a definite time period which not only attains highest degree of fusion but also causes minimal cell membrane fragility by achieving limited PEG exposure. It is speculated that highest degree of fusion would achieve better HCV replication in fused cell culture.

Figure 4.3 illustrates the findings of experiment to look at degree of fusion observed at different durations of PEG incubation. Dye labelled monocytes (Dil labelled) and Huh 7.5 cells (DiO labelled) were incubated in PEG for 1 to 3 minutes and plated for 24 hours according to optimised procedure discussed in previous section (Section 4.3.2, part C). After 24 hours cells were extracted to determine percentage of fused cells at different PEG incubation times through FACS analysis.

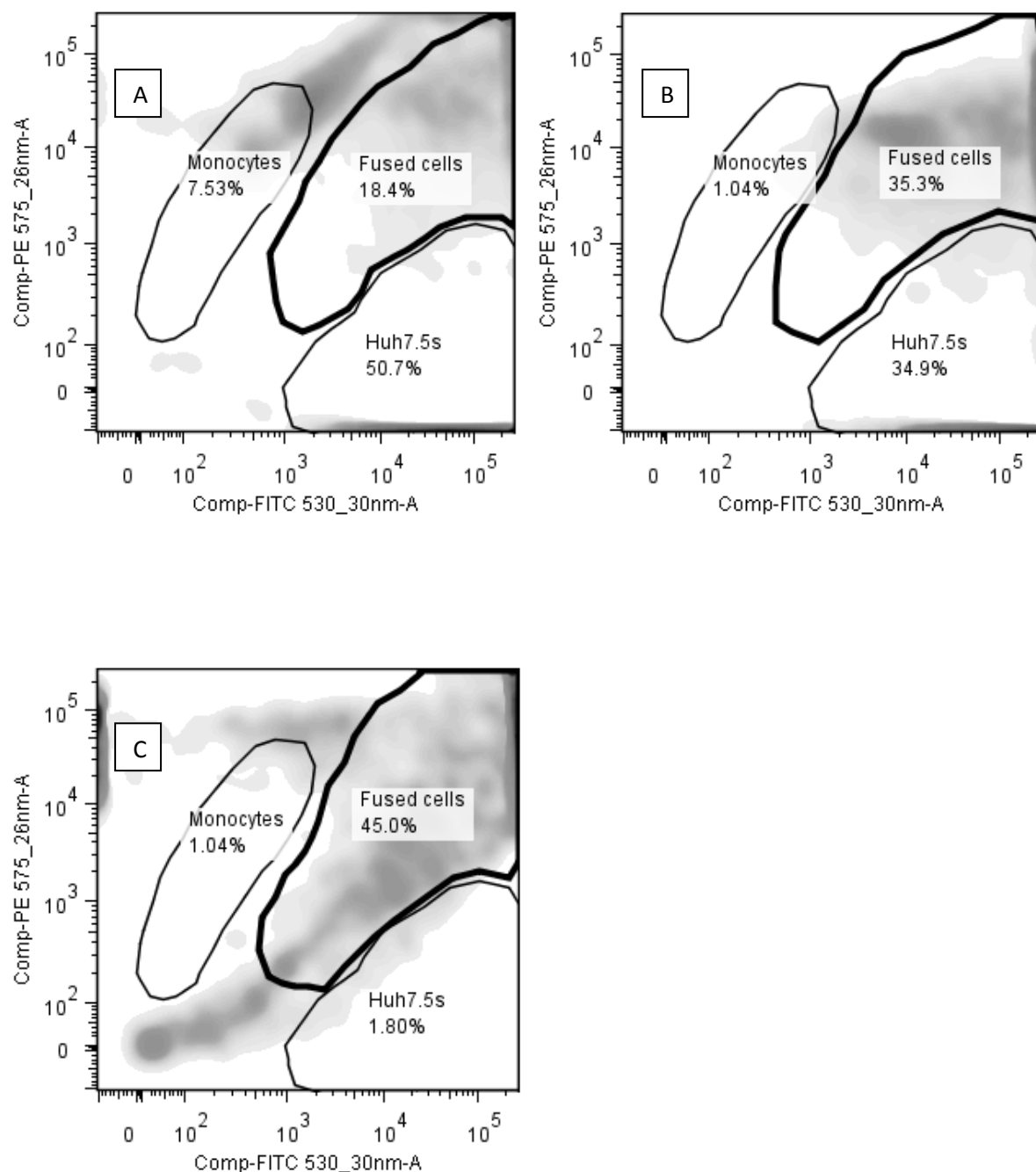


Figure 4.3: Extent of fusion depending upon the amount of time in PEG 1500: Percentage of fused cells after incubation in PEG for 1 minute (A), 2 minutes (B) and 3 minutes (C). Monocytes and Huh 7.5s were fused at 1:1 ratio.

According to the experiment above the proportion of fused cells after 1 minute incubation was 18.4 %, after 2 minutes incubation was 35.3% and after 3 minutes incubation was 45.0%, suggesting that increasing the duration of fusion increased the proportion of cells that fused. The proportion of cells that fused was determined in further experiments by extending PEG incubation time to 7 minutes. Figure 4.4 shows representative experiment from experiments done on three separate occasions.

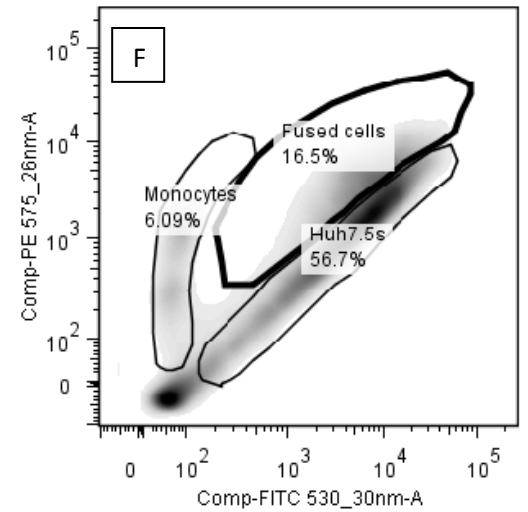
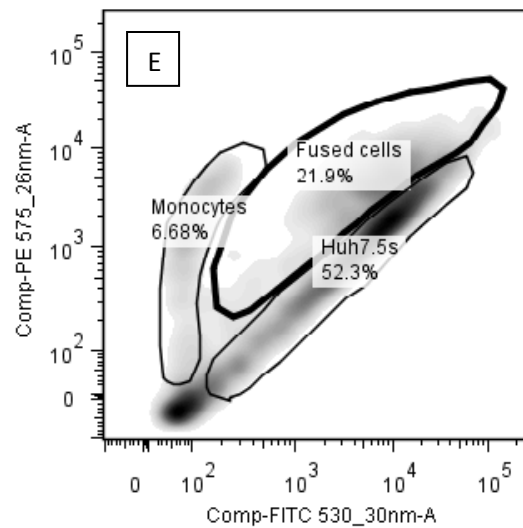
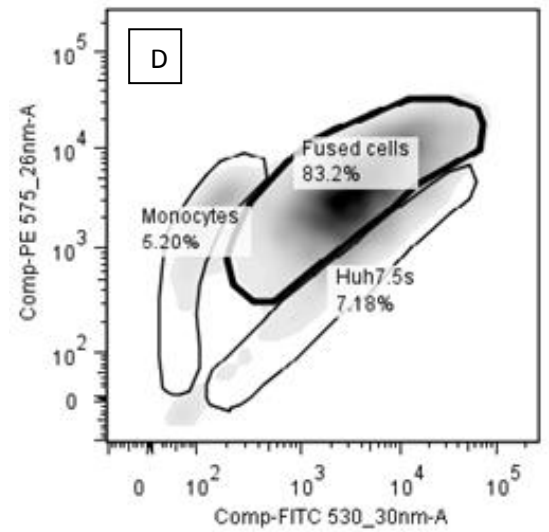
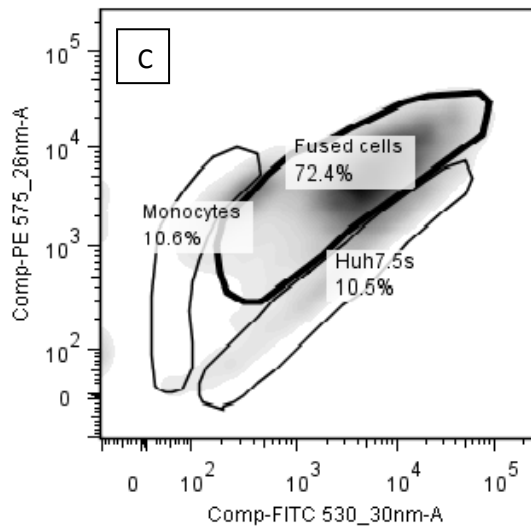
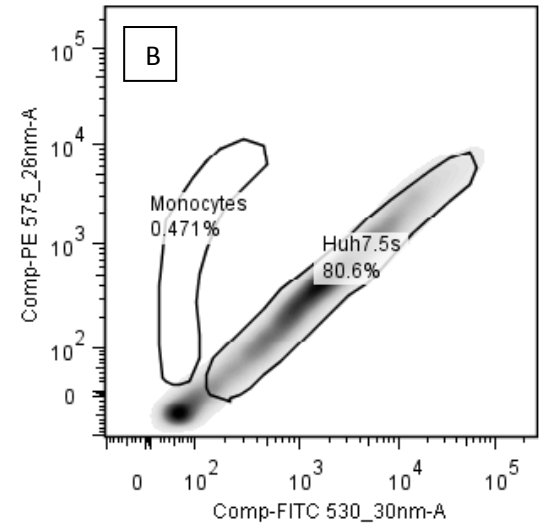
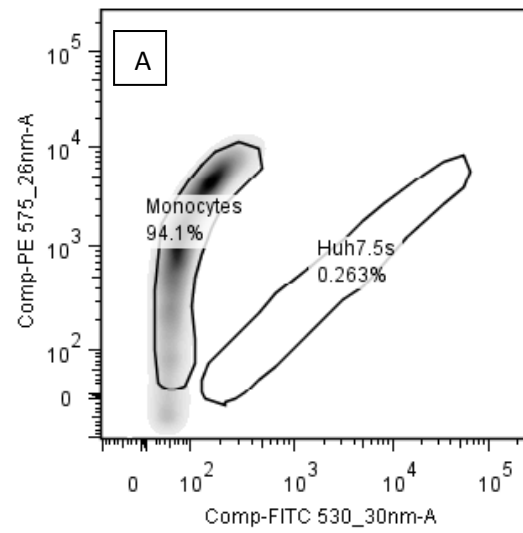


Figure 4.4: Degree of fusion depending upon the duration in PEG 1500: (A) and (B) represent monocyte and Huh 7.5s controls respectively. Percentage of fused cells after incubation in PEG for a definite time have been shown with 2 minutes (C), 3 minutes (D), 5 minutes (E) and 7 minutes (F) (Representative example from 3 separate experiments). Monocytes and Huh 7.5s were fused at 1:1 ratio.

The extent of fusion was observed at 2, 3, 5 and 7 minutes and the mean percentage of fusion from 3 different experiments was 52.36% at 2 minutes, 54.1% at 3 minutes, 16.86 % at 5 minutes and 16.61% at 7 minutes. This experiment was repeated on three separate occasions and the trend was consistent.

It was observed that cells incubated in PEG 1500 for more than 3 minutes showed massive cell death in the next 24 hours possibly because of excessive fragility of cell membrane induced due to prolonged period of PEG 1500 exposure.

E. Optimal pH conditions for fusion

DiO and Dil labelled Huh 7.5 cells and monocytes were exposed to PEG 1500 with pHs of 6, 7 and 8. The percentage of fusion were analysed by FACS. Figure 4.5, shows an example of amount of fusion between monocytes and Huh 7.5 cells at different pHs of PEG. The duration of the fusion was kept constant at 3 minutes in all experiment and ratio of monocytes and Huh 7.5 cells was 1:1.

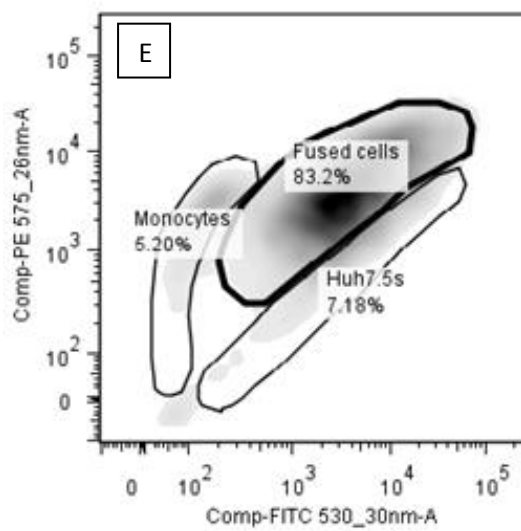
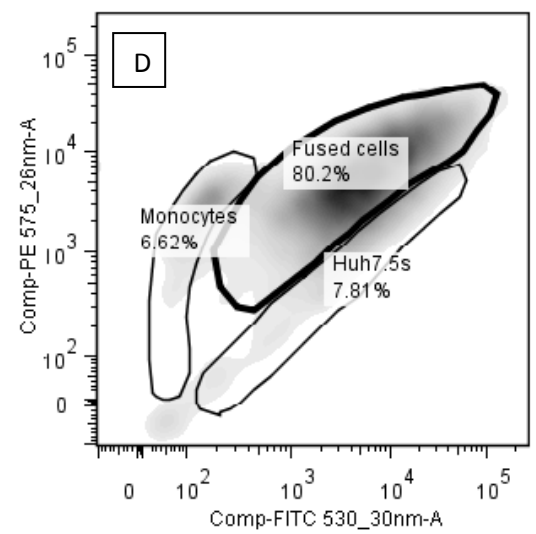
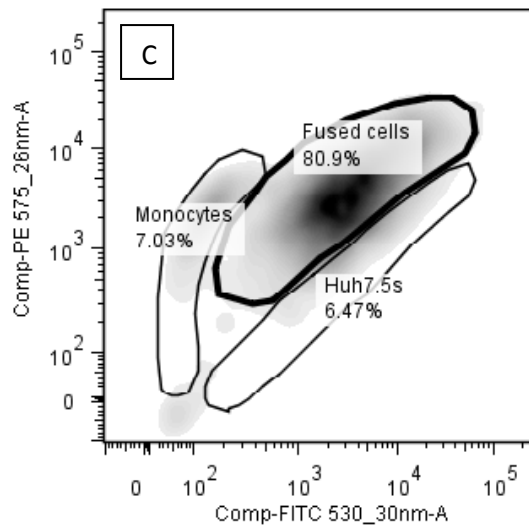
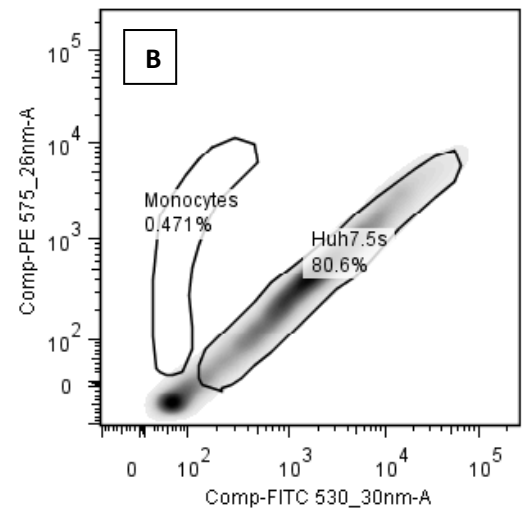
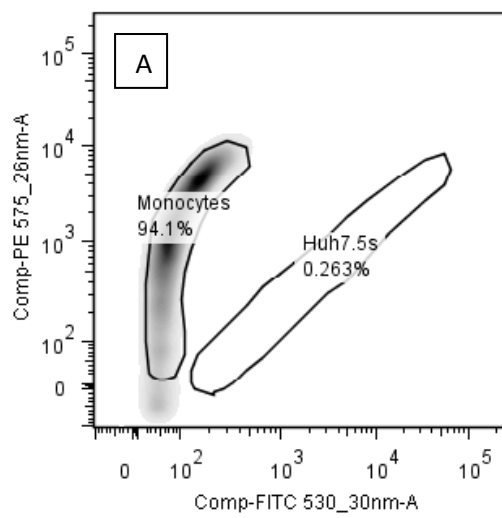


Figure 4.5: Relationship between the amount of fusion and the pH of PEG 1500: Monocyte (A), huh7.5s (B), PEG pH 6 (C), PEG pH 7 (D), PEG pH 8 (E) (Typical example from three separate experiments). Monocytes and Huh 7.5s were fused at 1:1 ratio.

Three separate experiments were done and it was observed that the trend was similar among all three. Mean percentage of fusion with PEG with pH of 6 was 53.61%, pH of 7 was 54% and pH of 8 was 53.6% (n=3).

F. Longitudinal measurement of fused cell frequency

After fusing dye labelled cells in the above favourable conditions, i.e. 1:1 monocytes and Huh 7.5 cell ratio, PEG 1500 with pH of 7, 3 minutes incubation in PEG 1500, using a large number of fused cells (0.5×10^{-6} per well) and small amount of DMEM with 10% FCS (500 μ l) in each well after fusion for first 24 hours, frequency and survival of persistently fused cells was observed at various times by growing the cells for several days and calculating the percentage of fused cells by flow cytometry at days 0, 3, 7 and 10. At day 3 cells were not CD14 bead selected as at that time Huh 7.5 cells do not overgrow fused cells probably because they are still in a state of recovery from the PEG 1500 insult. Massive overgrowth of unfused Huh7.5 cells was noted after day 5 and therefore it was chosen to select 'fused cells' (likely to contain replicating HCV) by CD14 bead selection (note that the primary monocytes which express CD14 do not proliferate in culture and therefore are unlikely to make a significant contribution to the expansion of CD14 expressing cells). The percentage of fused cells at these days was assessed by FACS analysis. It was seen that immediately after fusion fused

cells represented 30.7% of cells, at day 3 they were 26.5%, and the proportion of fused cells expressing CD14 at day 7 and 10 were 87.6% and 62.2% respectively after CD14 bead extraction. Please see figures 4.6 and 4.7 below.

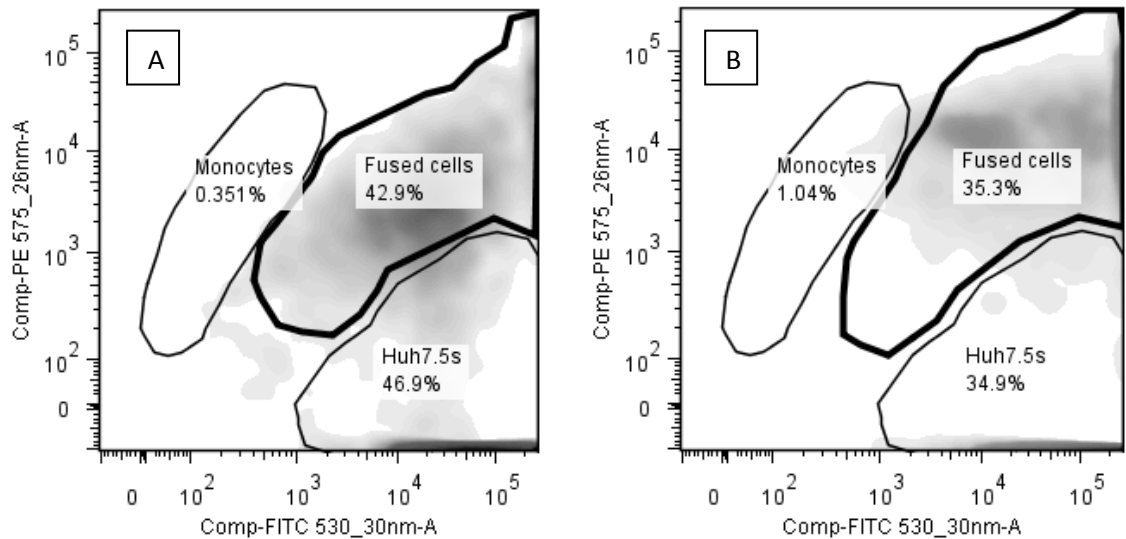


Figure 4.6: Frequency of fused cells immediately after fusion (A) and at day 3 (B). Monocytes and Huh 7.5s were fused at 1:1 ratio.

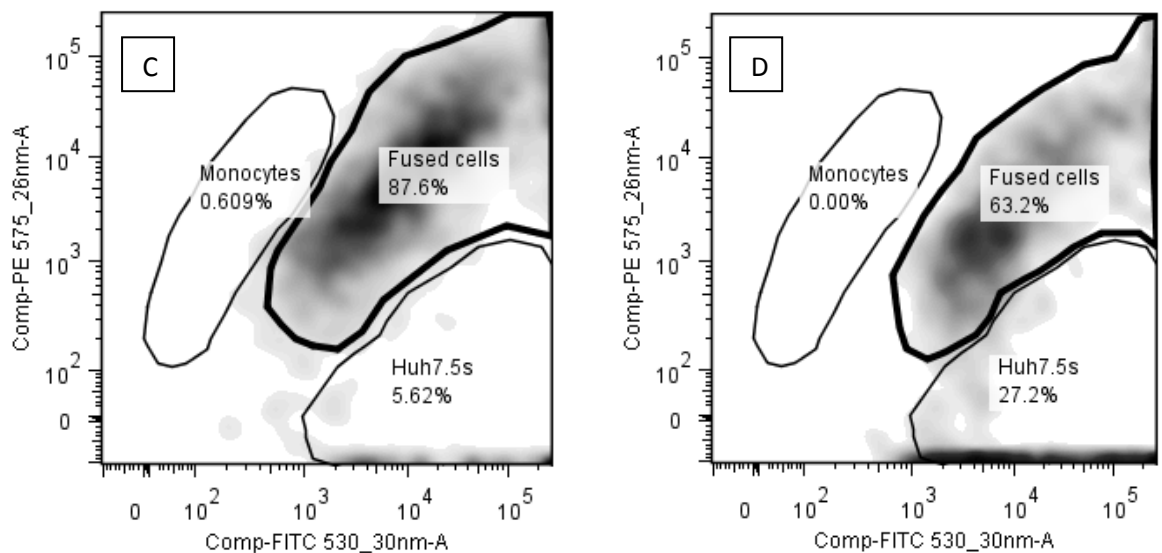


Figure 4.7: Frequency of fused cells after CD14 microbeads selection at day 7 (C) and 10 (D). Monocytes and Huh 7.5s were fused at 1:1 ratio.

These experiments indicated that significant numbers of fused cells could be generated and maintained in culture for at least 10 days using these optimised conditions. These experiments were not repeated further as it was determined that further optimisation was best performed using detection of replicating HCV by the detection methods. Overall using our optimised conditions the mean percentage of fused cells assessed immediately after fusion with PEG incubation of 3 minutes was 48.25% (Range 35.0% to 84.1% and n=10).

4.3.3. HCV expression analysed in fused cell lines by real time quantitative polymerase chain reaction

In order to confirm that HCV retained by monocytes in chronic HCV patients contained viable virions, monocytes fused with Huh 7.5 cells were assessed further for HCV RNA expressions by real time qPCR. Monocytes from chronic HCV patients were used to assess *in vitro* HCV expression after fusion with Huh 7.5 cells and culturing hybrid cell line for varying lengths of time. RNA from cells was extracted in trizol and the amount of HCV in the cells was quantified after reverse transcription of extracted RNA and real time PCR (See Materials and Methods). qPCR results were analysed by two types of relative quantification methods. In one method HCV infection was expressed as gene expression calibrated to β -actin while the other method was delta-delta C_T method in which HCV gene expression was calibrated

against β -actin and normalized with the positive control (cells containing HCV replicon). It was observed that the trend was the same with both the methods. Results here are given by the first method and expressed as HCV/ β -actin ratio. Log2 scale was used to show HCV RNA expression in graphs unless stated otherwise.

Replicon cells were used as a positive control while Huh 7.5 cells and water were used as negative controls for PCR. Firstly, RNA from cells was extracted from fusions for real time PCR analysis in the first week at different days, i.e. day 3, 5 and 7. Expression of HCV in unfused monocytes was also assessed by real time PCR and was used as a control to be assessed against the HCV expression in the fused population. HCV expression was also observed for longer periods of time in later experiments in a few patients.

4.3.3.1. HCV expression in fused cells (days 1 to 7)

In this section early pilot experiments to evaluate the feasibility of the approach are described and an account of fused cell populations, grown for different lengths of time till day 7 is given. A total of twenty four patients were studied, twelve belonged to genotype 1 and the other twelve were infected with the genotype 3 strain of HCV. The details of patients (ages, genotypes +/- subtypes, viral loads nearest to the time blood was taken for fusion, fibrosis scores if liver biopsy was done) are given in table 4.1 (patients infected with genotype 1 strain) and table 4.2 (patients infected with genotype 3 strain).

Patients infected with genotype 1 strain of HCV:

No	Date blood taken	Age	Genotype	Viral load	Fibrosis
1	29/07/2009	56	1a	5.93 log IU, 858376 IU	ND
2	16/10/2009	33	1b	6.89 log IU, 7714335 IU	ND
3	27/01/2010	38	1b	7.15 log IU, 14287115 IU	3
4	26/04/2010	58	1a	7.01 log IU, 10280828 IU	3
5	07/02/2011	61	1a	6.46 log IU, 2866058 IU	3-4
6	01/03/2011	56	1	5.91 log IU, 815485 IU	1
7	01/03/2011	59	1a	5.94 log IU, 870964 IU	ND
8	20/04/2011	42	1	5.60 log IU, 396661 IU	2
9	20/04/2011	47	1a	6 log IU, 1000000 IU	2
10	27/04/2011	53	1a	7.20 log IU, 16017910 IU	2
11	04/05/2011	55	1	6 log IU, 1000000 IU	ND
12	11/05/2011	50	1b	NA	6

Table 4.1: Details of genotype 1 patients used in early fusion experiments to look at HCV gene expression in fused monocytes and Huh 7.5 cells. The table shows age, genotype and subtype (if available), fibrosis score (Ishak scoring) if liver biopsy was done, viral load done closest to the time of blood sampling,

for each patient. NA= viral load not available at the time the blood sample was taken.

Figure 4.8, shows experiments performed with monocytes extracted from the blood samples of patients infected with genotype 1 strain of HCV. Fused monocytes and Huh 7.5 cells were selected out on different days particularly on days 3, 5 and 7 and HCV RNA expression was studied by using real time qPCR and quantified by calibrating HCV gene expression against that of β -actin. HCV RNA expression in replicon containing cells and monocytes are shown for comparison. The mean HCV viral load in these patients was 6.37 logIU/ml (range: 5.60 to 7.20 logIU/ml).

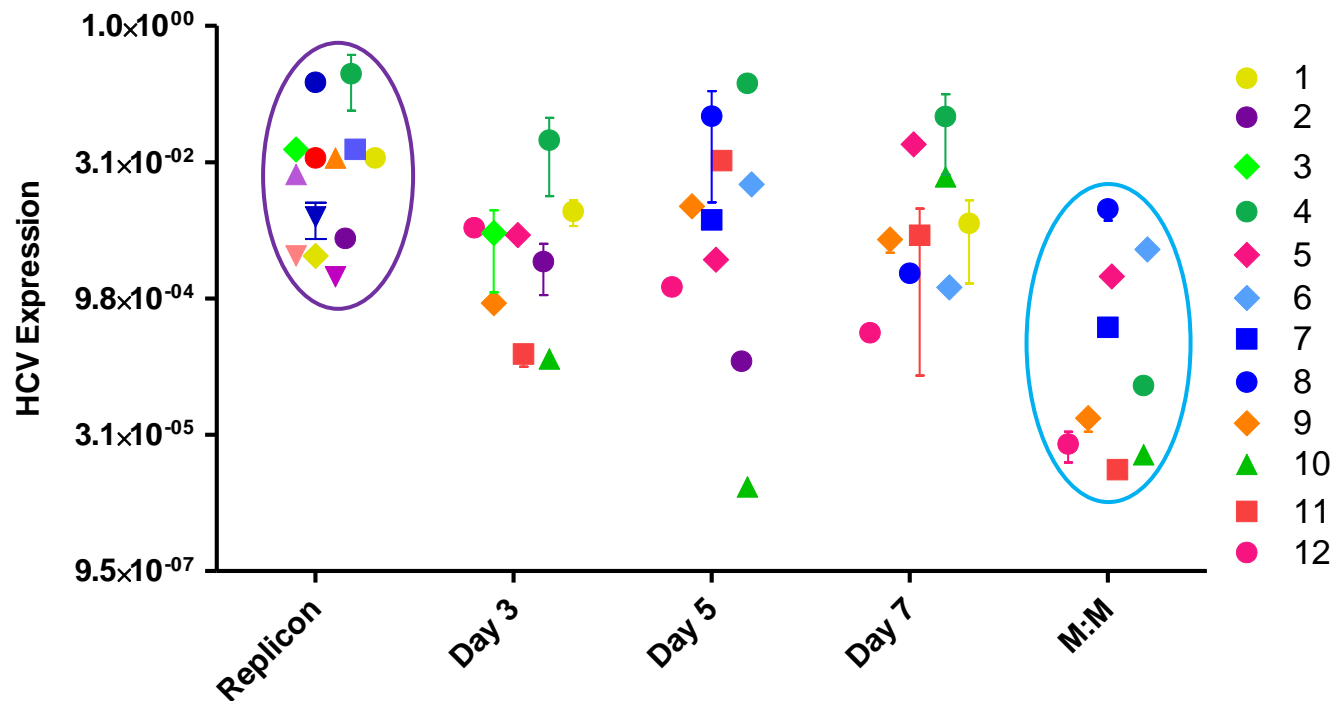


Figure 4.8: HCV expression in fused cells, using monocytes obtained from genotype 1 patients, cultured for varying lengths of time during the first week after fusion and extracted for the assessment of HCV RNA on different days: HCV infection is expressed as HCV gene expression calibrated to β -actin at various time periods in days (day 3 (n=9), 5 (n=10) and 7 (n=9)). Replicon cells circled in purple on the left side acted as the positive control for fusion experiments. HCV expression in monocytes from different patients is circled in blue on the right side. Each point represents the mean of samples from a single patient run in duplicate during real time qPCR. Patient number with colour coding is given on the right hand side.

Patients infected with genotype 3 strain of HCV:

No	Date blood taken	Age	Genotype	Viral load	Fibrosis
1	28/05/2009	66	3	3.02 log IU, 1036 IU	ND
2	01/07/2009	47	3a	4.77 log IU, 59330 IU	1
3	05/08/2009	48	3	6.57 log IU, 3720254 IU	1
4	02/09/2009	56	3a	6.65 log IU, 4417016 IU	2
5	16/09/2009	44	3	4.99 log IU, 98467 IU	2
6	02/11/2009	35	3a	6.78 log IU, 5968257 IU	5
7	19/01/2011	30	3a	5.16 log IU, 142918 IU	2
8	18/02/2011	57	3a	5.19 log IU, 153103 IU	4
9	18/05/2011	44	3a	6 log IU, 1000000 IU	5
10	18/05/2011	38	3b	6 log IU, 1000000 IU	2
11	25/05/2011	57	3a	6.23 log IU, 1699933 IU	ND
12	25/05/2011	59	3b	5 log IU, 100000 IU	ND

Table 4.2: Details of genotype 3 patients used in early fusion experiments to look at HCV gene expression in fused monocytes and Huh 7.5 cells. The table shows age, genotype and subtype (if available), fibrosis score (Ishak scoring)

if liver biopsy was done, The viral load test performed at the nearest point in time to the biopsy was recorded.

Figure 4.9, shows experiments performed with monocytes extracted from the blood samples of patients infected with genotype 3 strain of HCV. Fused monocytes and Huh 7.5 cells were selected out on different days particularly day 3, 5 and 7 and HCV RNA expression was studied by using real time qPCR and quantified by calibrating HCV gene expression against that of β -actin. HCV RNA expression in replicon containing cells and monocytes are shown for comparison. The mean HCV viral load in these patients was 5.53 logIU/ml (range: 3.02 to 6.78 logIU/ml).

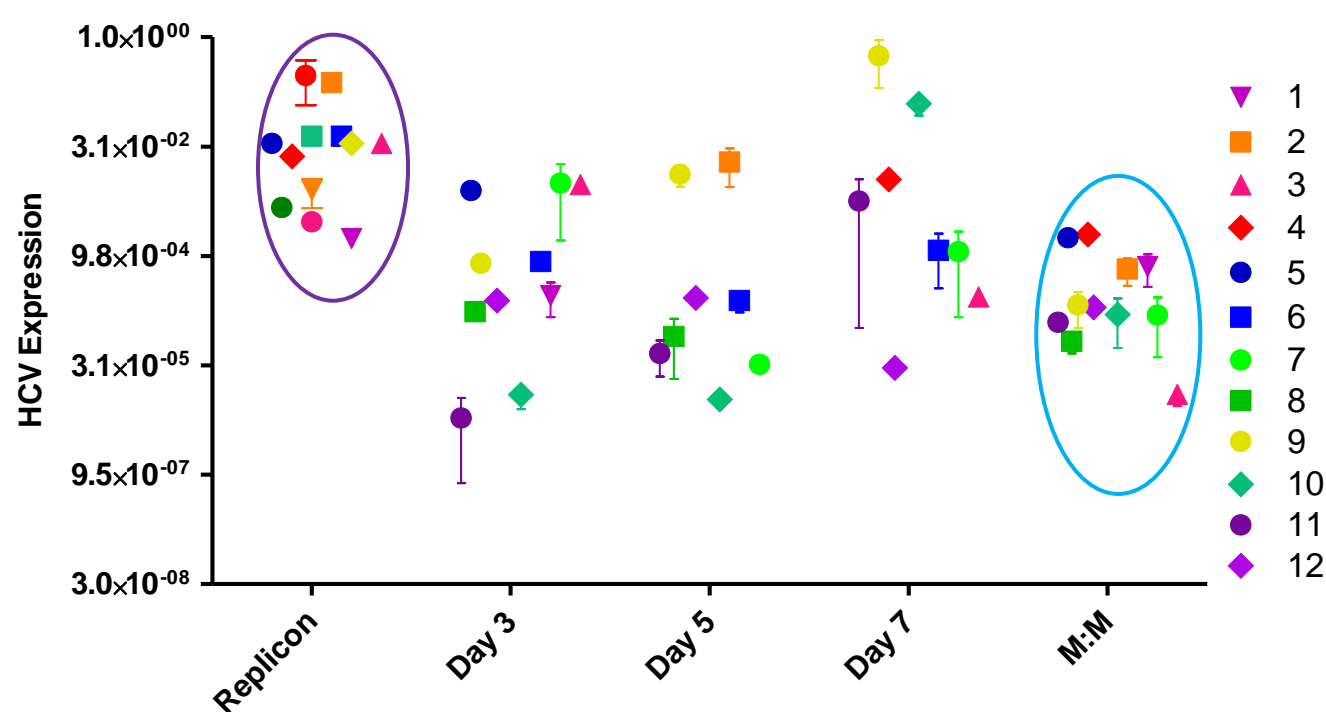


Figure 4.9: HCV expression in fused cells, using monocytes obtained from genotype 3 patients, cultured for varying lengths of time during the first week and extracted for assessment of HCV RNA on different

days: HCV infection is expressed as HCV gene expression calibrated to β -actin at various time periods in days (day 3 (n=10), 5 (n=8) and 7 (n=8)). Replicon cells circled in purple on the left side is positive control for fusion experiments. HCV expression in monocytes from different patients is circled in blue on the right side. Each point represents mean of sample from a single patient run in duplicate during real time PCR. Patient number with colour coding is given on the right hand side. HCV expression represents the HCV RNA: β -actin ratio (calculated by the relative quantification method).

The above experiments suggested that 3-7 days after fusion HCV expression in fused cells was increased in most fused cell cultures both in genotype 1 and 3 compared to expression in monocytes. This suggests the presence of HCV replication in fused cell cultures.

To confirm our initial observations HCV gene expression in monocytes of 19 patients was compared to HCV RNA in their monocytes fused with Huh 7.5 cells. The details of patients whose monocytes were compared with fused cultures derived by fusing monocytes from same patients with Huh 7.5 cells are given in table 4.3 shown below.

No	Date blood taken	Age	Genotype	Viral load	Fibrosis
1	26/04/2010	58	1a	7.01 log IU, 10280828 IU	3
2	07/02/2011	61	1a	6.46 log IU, 2866058 IU	3-4
3	01/03/2011	56	1	5.91 log IU, 815485 IU	1
4	01/03/2011	59	1a	5.94 log IU, 870964 IU	ND
5	20/04/2011	42	1	5.60 log IU, 396661 IU	2
6	20/04/2011	47	1a	6 log IU, 1000000 IU	2
7	27/04/2011	53	1a	7.20 log IU, 16017910 IU	2
8	04/05/2011	55	1	6 log IU, 1000000 IU	ND
9	11/05/2011	50	1b	NA	6
10	28/05/2009	66	3	3.02 log IU, 1036 IU	ND
11	01/07/2009	47	3a	4.77 log IU, 59330 IU	1
12	02/09/2009	56	3a	6.65 log IU, 4417016 IU	2
13	16/09/2009	44	3	4.99 log IU, 98467 IU	2
14	19/01/2011	30	3a	5.16 log IU, 142918 IU	2
15	18/05/2011	44	3a	6 log IU, 1000000 IU	5
16	18/05/2011	38	3b	6 log IU, 1000000 IU	2

17	25/05/2011	57	3a	6.23 log IU, 1699933 IU	ND
18	25/05/2011	59	3b	5 log IU, 100000 IU	ND
19	18/02/2011	57	3a	5.19 log IU, 153103 IU	4

Table 4.3: Details of patients whose HCV RNA expression in monocytes was compared with their fused cells. The table shows age, genotype and subtype (if available), fibrosis score (Ishak scoring) if liver biopsy was done, viral load done closest to the time of blood sampling from each patient. NA = viral load not available at the time blood sample was taken.

Monocytes from patients were separated into two populations some were used in the fusion process and 1×10^6 monocytes were left in culture for 3 days initially to compare replication in unfused monocytes with replication in fused cells. However by day 3 of the culture most (>80% by trypan blue exclusion) of the monocytes were dead and thus a comparison of HCV in monocytes cultured for 3 days was not possible. We therefore compared HCV RNA levels in monocytes immediately after CD14 bead separation and RNA was extracted from the cells and used for qPCR analysis. The remainder of the isolated monocytes were fused to Huh 7.5 cells. Each experiment was performed in duplicate and the mean HCV viral load of these patients was 5.73 logIU/ml (range: 3.02 to 7.20 logIU/ml). Figure 4.10 shows the results obtained by comparing HCV RNA expression in fused cells with the unfused monocytes.

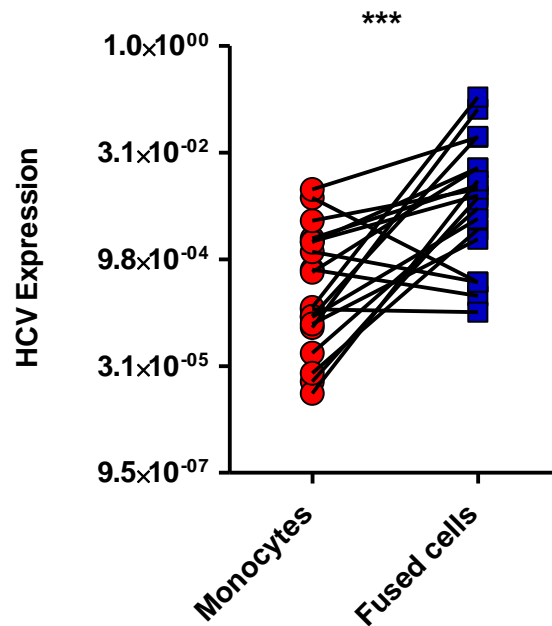


Figure 4.10: Comparison of HCV RNA expression in monocytes and fused Cells: HCV expression in monocytes is compared with fused cells (each point represents average HCV expression taken from all cultures isolated at different days from one patient) (p=0.0016). HCV expression represents the HCV RNA: β -actin ratio (calculated by the relative quantification method).**

HCV expression was significantly higher in fused cells compared to that in the monocytes ($p=0.0016$). Out of 19 patients, 15 showed a rise in HCV RNA expression in fused cells compared to their monocytes. For every patient average HCV RNA expression was considered at different days.

In order to look at HCV RNA expression separately at different days in comparison to monocytes, HCV expression of fused cells isolated on days 3, 5 and 7 was compared with HCV expression in the monocytes of that patient. Some patients' fused cells were isolated from culture for HCV RNA qPCR analysis at all 3 days while some were isolated at less than three occasions, either for any 2 days or isolated only once on either of the above mentioned days. Out of 19 patients 15 patients' fused cells were isolated on day 3 and their details are given in table 4.4.

No	Date blood taken	Age	Genotype	Viral load	Fibrosis	Fold Change
1	26/04/2010	58	1a	7.01 log IU, 10280828 IU	3	512
2	07/02/2011	61	1a	6.46 log IU, 2866058	3-4	2.9
3	20/04/2011	47	1a	6 log IU, 1000000 IU	2	11.5
4	27/04/2011	53	1a	7.20 log IU, 16017910 IU	2	18.7
5	04/05/2011	55	1	6 log IU, 1000000 IU	ND	19.00
6	11/05/2011	50	1b	NA	6	246
7	28/05/2009	66	3	3.02 log IU, 1036 IU	ND	-2.5
8	01/07/2009	47	3a	4.77 log IU, 59330 IU	1	29.6
9	16/09/2009	44	3	4.99 log IU, 98467 IU	2	4.44
10	19/01/2011	30	3a	5.16 log IU, 142918 IU	2	1.24
11	18/05/2011	44	3a	6 log IU, 1000000 IU	5	3.75
12	18/05/2011	38	3b	6 log IU, 1000000 IU	2	65.3
13	25/05/2011	57	3a	6.23 log IU, 1699933 IU	ND	-12.7
14	25/05/2011	59	3b	5 log IU, 100000 IU	ND	-20.65
15	18/02/2011	57	3a	5.19 log IU, 153103 IU	4	2.53

Table 4.4: Details of patients whose HCV RNA expression in monocytes was compared with their fused cells isolated on day 3. The table shows age, genotype and subtype (if available), fibrosis score (Ishak scoring) if liver biopsy was done, viral load done closest to the time of blood sampling from each patient and fold change in HCV RNA after fusion compared to HCV RNA in monocytes. NA = viral load not available at the time when blood sample was taken.

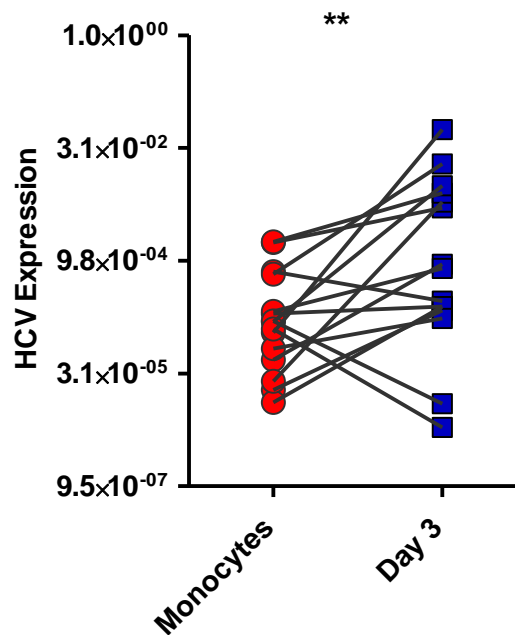


Figure 4.11: Comparison of HCV RNA expression in monocytes and fused cells at day 3. (p=0.0067 using Wilcoxon matched pair test). Monocytes were processed straight after CD14 bead extraction. HCV expression represents the HCV RNA: β -actin ratio (calculated by the relative quantification method).**

12 patients showed a rise in HCV expression in fused cells compared to their monocytes in cells isolated at day 3.

Out of 19 patients 15 patients' fused cells were isolated on day 5 and their details are given in table 4.5.

No	Date blood taken	Age	Genotype	Viral load	Fibrosis	Fold Change
1	26/04/2010	58	1a	7.01 log IU, 10280828 IU	3	2167
2	07/02/2011	61	1a	6.46 log IU, 2866058	3-4	1.55
3	01/03/2011	56	1	5.91 log IU, 815485 IU	1	5.26
4	01/03/2011	59	1a	5.94 log IU, 870964 IU	ND	-2.7
5	20/04/2011	42	1	5.60 log IU, 396661 IU	2	10.63
6	20/04/2011	47	1a	6 log IU, 1000000 IU	2	-2.22
7	27/04/2011	53	1a	7.20 log IU, 16017910 IU	2	218.5
8	04/05/2011	55	1	6 log IU, 1000000 IU	ND	260
9	11/05/2011	50	1b	NA	6	54.8
10	19/01/2011	30	3a	5.16 log IU, 142918 IU	2	1.32
11	18/05/2011	44	3a	6 log IU, 1000000 IU	5	63.5
12	18/05/2011	38	3b	6 log IU, 1000000 IU	2	-4.73
13	25/05/2011	57	3a	6.23 log IU, 1699933 IU	ND	-3.8
14	25/05/2011	59	3b	5 log IU, 100000 IU	ND	-2.7
15	18/02/2011	57	3a	5.19 log IU, 153103 IU	4	1.16

Table 4.5: Details of the patients whose HCV RNA expression in monocytes was compared with their fused cells isolated on day 5. The table shows age, genotype and subtype (if available), fibrosis score (Ishak scoring) if liver biopsy was done, viral load done closest to time of the blood sampling from each patient and fold change in HCV RNA after fusion compared to HCV RNA in monocytes. NA= not available.

Figure 4.12, shows HCV expression in fused cells extracted on day 5 compared with that in monocytes. Monocytes were processed straight after CD14 bead extraction.

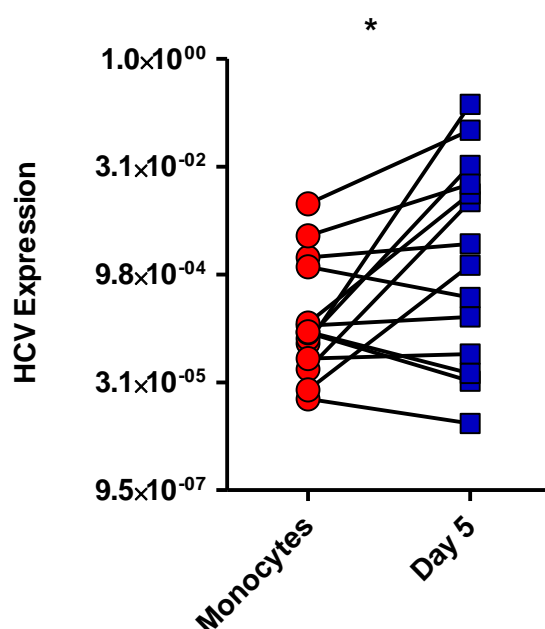


Figure 4.12: Comparison of HCV expression (HCV/ β -actin ratio) in monocytes and fused cells at day 5. (* $p=0.02$ using Wilcoxon matched pair test). HCV expression represents the HCV RNA: β -actin ratio (calculated by the relative quantification method).

11 patients showed an increase in HCV gene expression in fused cells isolated at day 5, compared to their monocytes

Out of 19 patients 14 patients' fused cells were isolated on day 7. Table 4.6 gives details of these patients.

No	Date blood taken	Age	Genotype	Viral load	Fibrosis	Fold Change
1	26/04/2010	58	1a	7.01 log IU, 10280828 IU	3	932
2	07/02/2011	61	1a	6.46 log IU, 2866058	3-4	29.02
3	01/03/2011	56	1	5.91 log IU, 815485 IU	1	-2.62
4	20/04/2011	42	1	5.60 log IU, 396661 IU	2	-5.14
5	20/04/2011	47	1a	6 log IU, 1000000 IU	2	1194
6	27/04/2011	53	1a	7.20 log IU, 16017910 IU	2	93.8
7	04/05/2011	55	1	6 log IU, 1000000 IU	ND	386
8	11/05/2011	50	1b	NA	6	17.1
9	02/09/2009	56	3a	6.65 log IU, 4417016 IU	2	5.8
10	19/01/2011	30	3a	5.16 log IU, 142918 IU	2	-6.9
11	18/05/2011	44	3a	6 log IU, 1000000 IU	5	2704
12	18/05/2011	38	3b	6 log IU, 1000000 IU	2	7.5
13	25/05/2011	57	3a	6.23 log IU, 1699933 IU	ND	794.4
14	25/05/2011	59	3b	5 log IU, 100000 IU	ND	46.6

Table 4.6: Details of the patients whose HCV RNA expression in monocytes was compared with their fused cells isolated on day 7. The table shows age, genotype and subtype (if available), fibrosis score (Ishak scoring) if liver biopsy was done, viral load done closest to the time of blood sampling from each patient and fold change in HCV RNA after fusion compared to HCV RNA in monocytes. ND= not done, NA= not available.

Figure 4.13, shows HCV expression in fused cells extracted on day 7 compared with that in monocytes. Monocytes were processed straight after CD14 bead extraction.

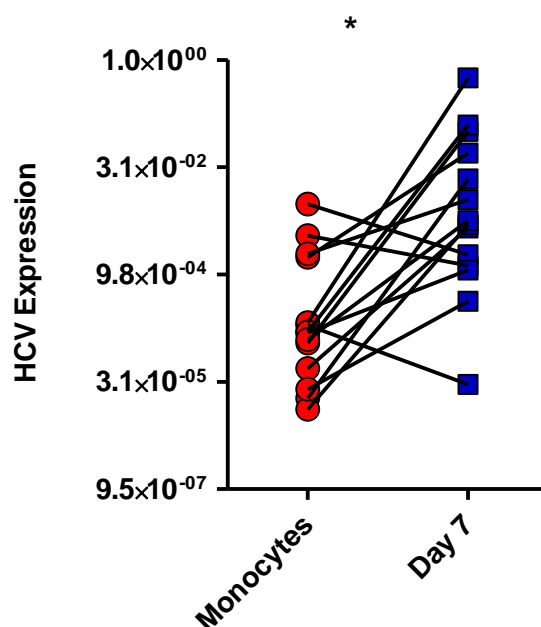


Figure 4.13: Comparison of HCV expression in monocytes and fused Cells at day 7. (*p=0.01 using Wilcoxon matched pair test). HCV expression represents the HCV RNA: β -actin ratio (calculated by the relative quantification method).

11 patients showed an increase in HCV expression in fused cells compared to the monocytes when isolated at day 7.

The next step was to assess HCV expression in fused cells in patient's monocytes cultured for longer than 7 days after fusion with Huh 7.5s.

4.3.3.2. HCV expression in fused cells cultured for more than 7 days

In some patients fused cells were grown for longer than seven days. This was to see whether a cell line infected with naturally occurring HCV derived from patients' monocytes could be generated. HCV expression was assessed in these patients and compared with HCV expression in fused cells cultured for less than seven days. Comparison of HCV expression in cultured cells between the 1 to 7 days compared to those isolated between 10-21 days is shown in figure 4.14. The mean HCV viral load of patients was 5.25logIU/ml (range: 3.02 to 6.65logIU/ml).

No	Date blood taken	Age	Genotype	Viral load	Fibrosis
1	28/05/2009	66	3	3.02 log IU, 1036 IU	ND
2	01/07/2009	47	3a	4.77 log IU, 59330 IU	1
3	05/08/2009	48	3	6.57 log IU, 3720254 IU	1
4	02/09/2009	56	3a	6.65 log IU, 4417016 IU	2

Table 4.7: Details of the patients whose monocytes were used in the fusion experiments to look at HCV gene expression in fused cells after a longer time scale. The table shows age, genotype and subtype (if available),

fibrosis score (Ishak scoring) if liver biopsy was done, The viral load test performed at the nearest point in time to the biopsy was recorded. ND= not done.

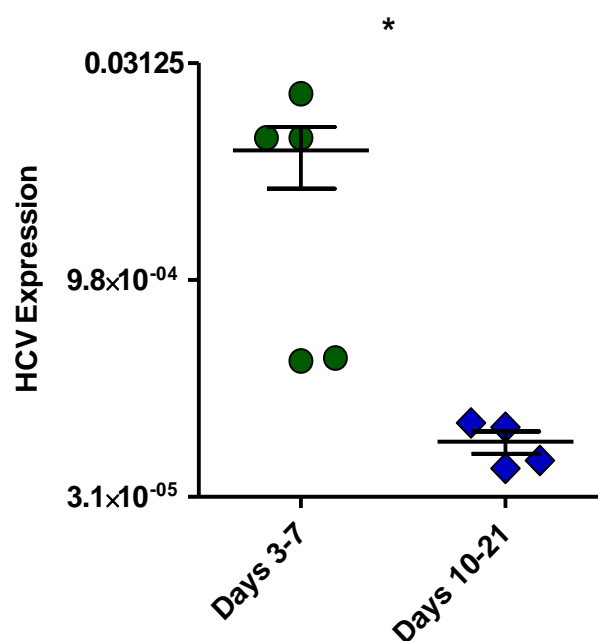


Figure 4.14: Comparison of HCV expression in fused cells cultured for more than 7 Days in four patients: Each point represents HCV expressions at a different day. For one patient HCV expression for both days 3 and 7 was available (*p=0.02 using Mann Whitney U test). HCV expression represents the HCV RNA: β -actin ratio (calculated by the relative quantification method).

These data suggest that fusing monocytes from patients with HCV with Huh 7.5 cells leads to increased expression of HCV for the first 7 days after fusion and a decline in HCV RNA thereafter, indicating HCV RNA replication for the

initial 7 days. Therefore, HCV could not be stably expressed in Huh 7.5 cells for longer duration in order to establish an HCV infected cell line.

4.3.4. Indirect immunofluorescence microscopy

4.3.4.1. Single staining with anti-NS5A

Hybrid cells obtained after fusion of HCV patients' monocytes with Huh 7.5 cells were extracted on 3rd, 5th and 7th days and stained for the HCV encoded protein NS5A using polyclonal sheep anti-NS5A (a kind gift from Professor Mark Harris, University of Leeds) as primary antibody while the secondary antibody was alexa-fluor 488 nm-conjugated polyclonal IgG (goat anti-sheep). Replicon cells were stained as positive controls (figure 4.15) and Huh 7.5 cells were taken as negative controls (figure 4.16). NS5A staining of fused cells and their controls are given below. Figure 4.17 shows the fused cells stained with NS5A antibody.

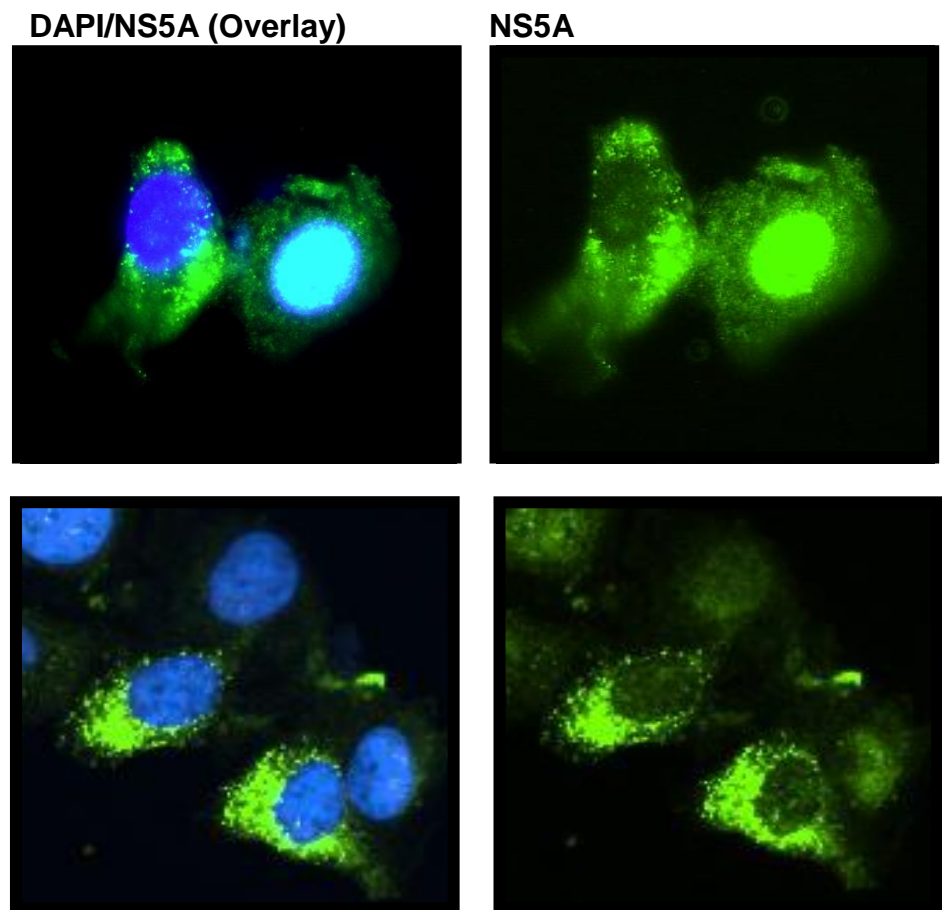


Figure 4.15: Replicon cells being used as positive control for this experiment, are stained with antibody against NS5A, using alexa-fluor 488-nm conjugated polyclonal IgG as a secondary antibody. On left are the overlays of DAPI and NS5A staining while on right are only NS5A stained cells (in this case staining positive)

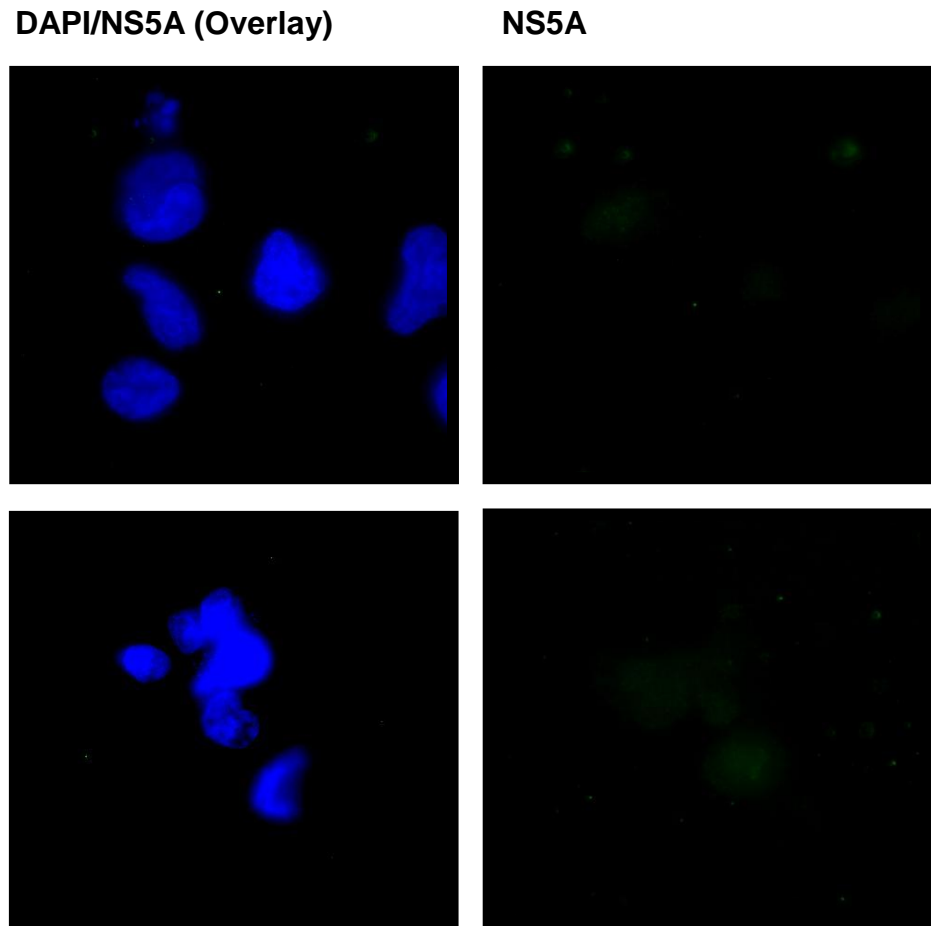
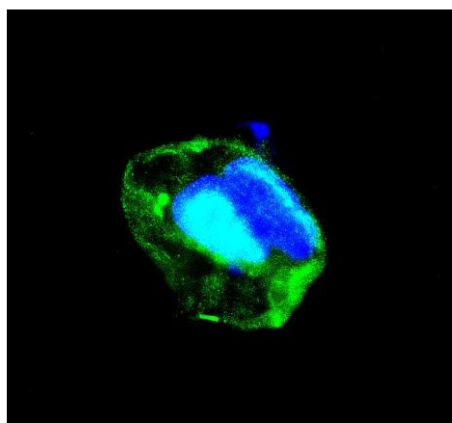
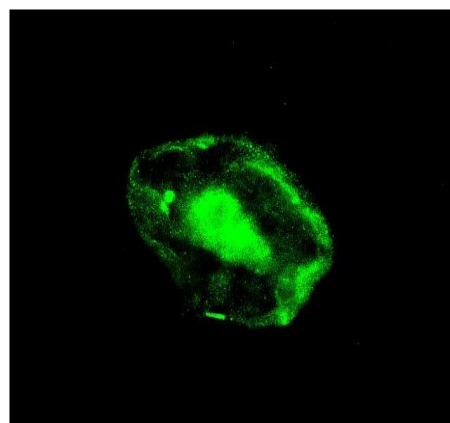


Figure 4.16: Huh 7.5 cells being used as negative control for this experiment, do not stain positive with antibody against NS5A, using alexa-fluor 488-nm conjugated polyclonal IgG as a secondary antibody. On left are the overlays of DAPI stain and NS5A staining while on right are only NS5A stained cells (in this case staining negative).

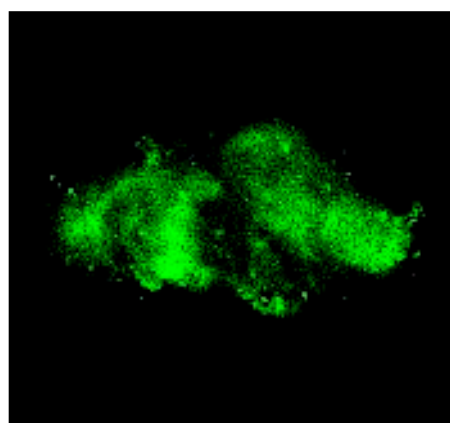
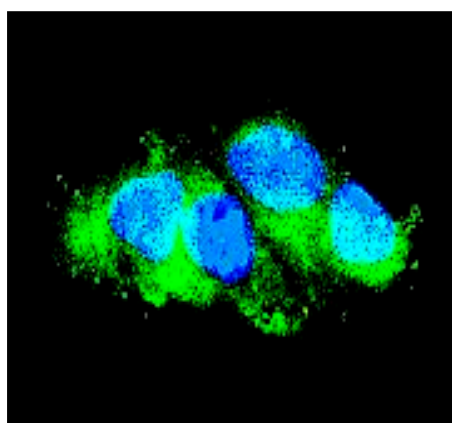
DAPI/NS5A (Overlay)



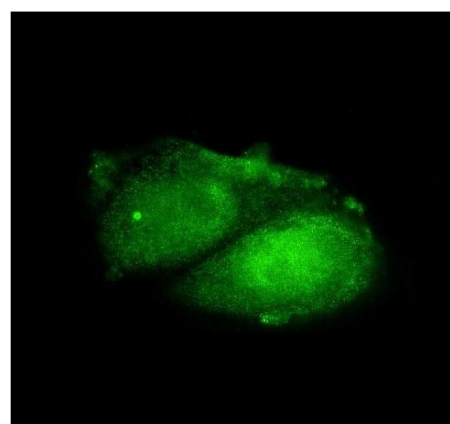
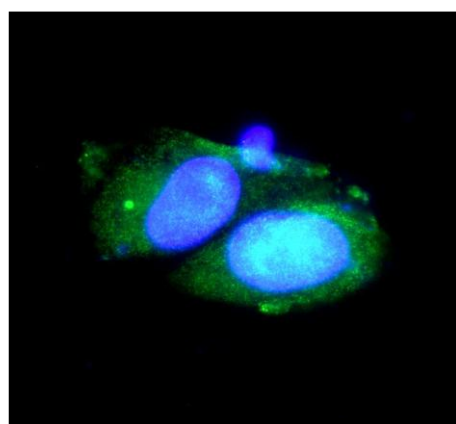
NS5A



Fusion at Day 3

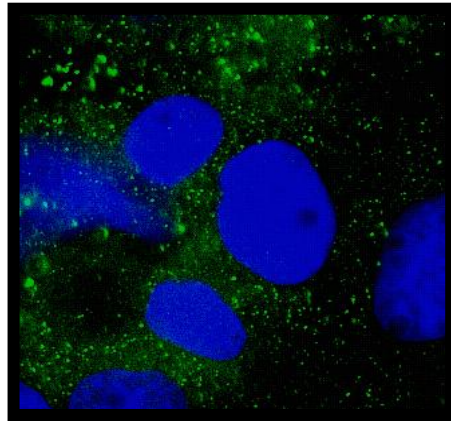


Fusion at Day 3

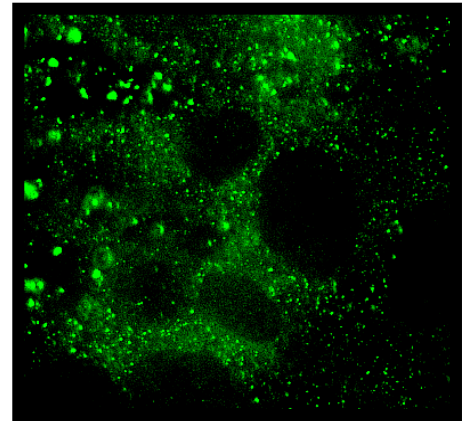


Fusion at Day 5

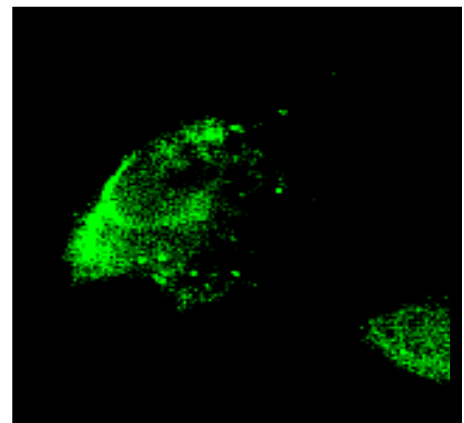
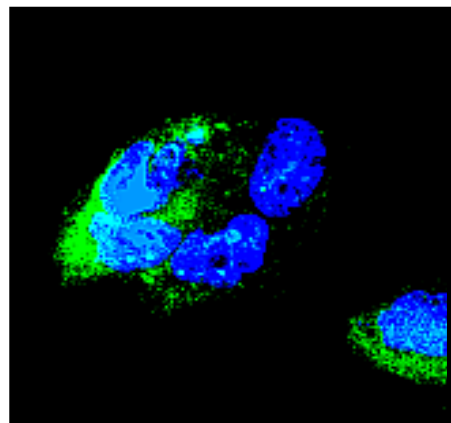
DAPI/NS5A (Overlay)



NS5A



Fusion at Day 5



Fusion at Day 7

Figure 4.17: Fused cells extracted on different days from different HCV patients and stained with antibody against NS5A, using alexa-fluor 488-nm conjugated polyclonal IgG as a secondary antibody. On left are the overlays of DAPI and NS5A staining while on right are only NS5A stained cells (in this case staining positive). (Monocytes in fused cells were derived from genotype 1b HCV patient and viral load was 6.89logIU/ml at the time when blood was taken, derived from genotype 3a HCV patient and viral load was 6.78logIU/ml at the time when blood was taken).

Fused cells stained positive for NS5A protein. NS5A is a non-structural phosphoprotein, composed of approximately 447 amino acids and is released during processing of viral polyprotein. It is involved in viral replication and assembly (He, Staschke et al. 2006). Its exact role in the replication process is poorly understood (Huang, Staschke et al. 2007). It has also been implicated in modulating cell signalling pathways, interferon response and pathogenesis and in regulating apoptosis. Moreover, it is thought to have an enzymatic function which is not very clear yet (Yamasaki, Arcuri et al. 2012). It is thought that it works by interacting with other HCV and host cellular proteins (He, Staschke et al. 2006, Reed, Xu et al. 1997, Macdonald and Harris 2004, Liang, Ye et al. 2007, Love, Brodsky et al. 2009). NS5A staining represents viral life cycle progression and replication process in fused cells.

4.3.4.2. Double staining with anti-NS5A and anti-albumin

To determine whether the fused cells that expressed HCV were genuinely hepatocytes rather than, for example, fused monocytes, antibodies directed against HCV and the liver specific marker albumin were used to stain hepatocytes containing loci of HCV replication. Fused cells from one patient were stained for NS5A using polyclonal sheep anti-NS5A as primary antibody while secondary antibody was alexa-fluor 488 nm-conjugated polyclonal IgG (goat anti-sheep). Replicon cells were stained as positive controls and Huh 7.5s were taken as negative control. After that cells were stained for albumin, which is a hepatocyte marker, with polyclonal rabbit anti-albumin (from DAKO, Glostrup, Denmark) to differentiate Huh 7.5s from

monocytes. The secondary antibody for anti-albumin was PE-conjugated mouse anti-rabbit (from Invitrogen Molecular Probes). Replicon cells and Huh 7.5s stained positive for albumin. Fused cells stained positive for both albumin and NS5A.

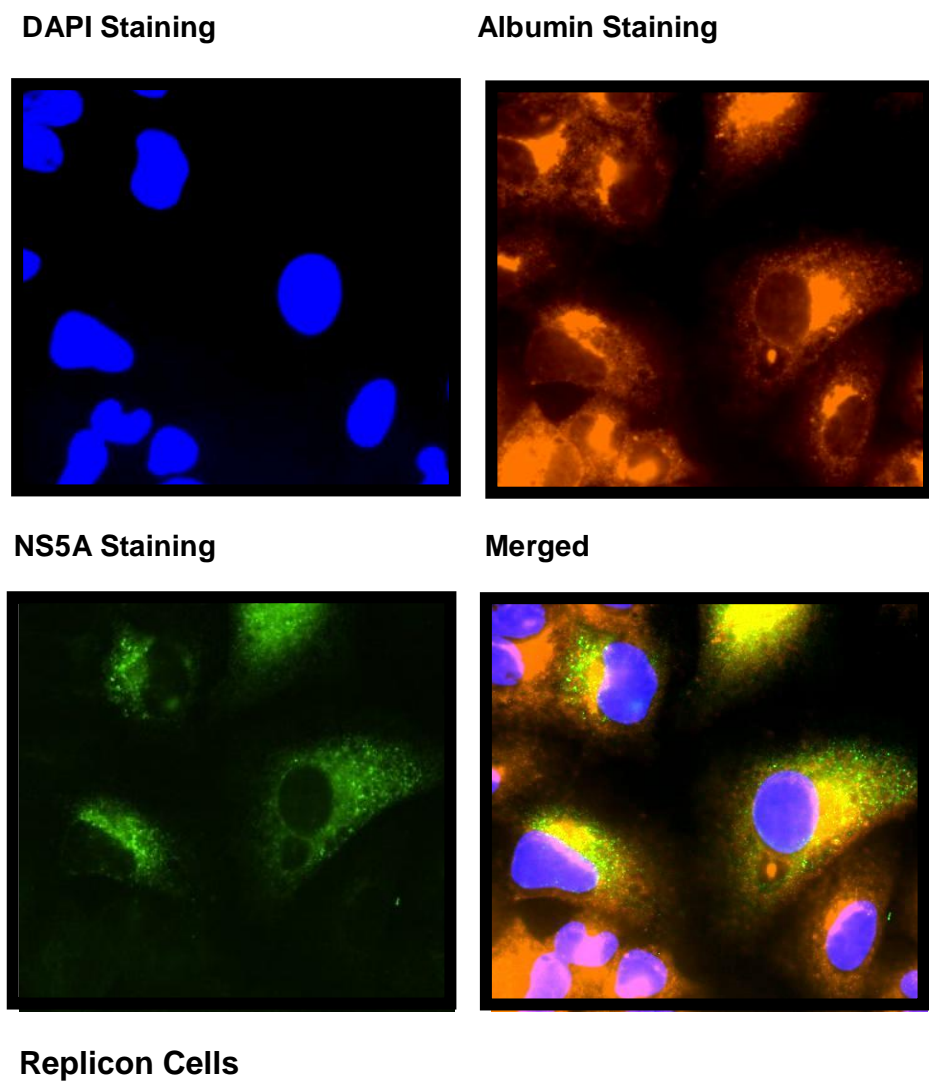
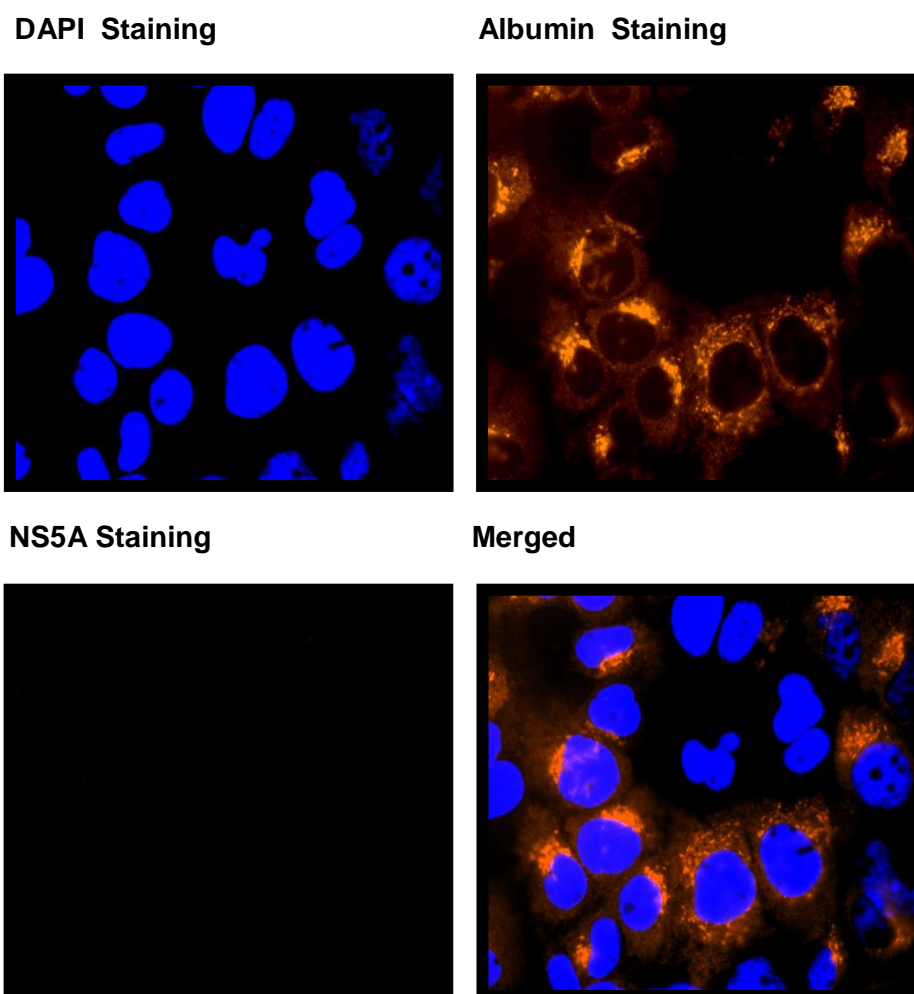


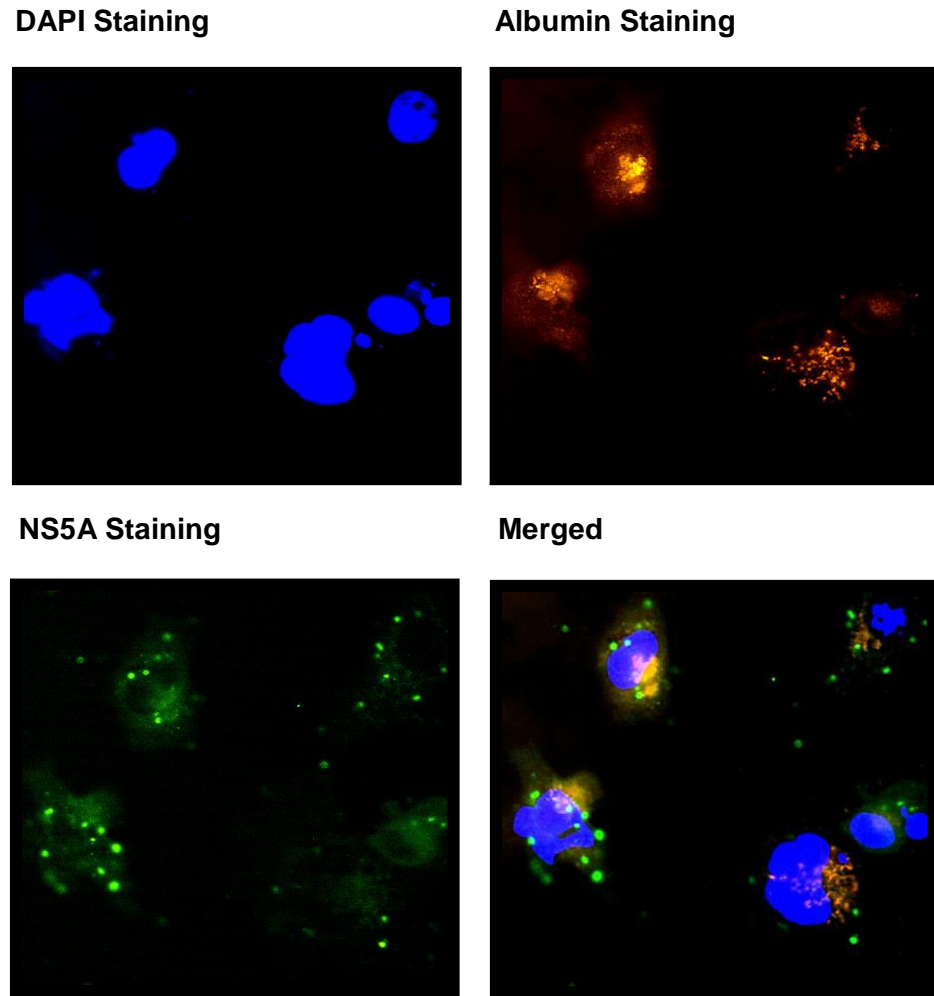
Figure 4.18: Dual staining of the replicon cells: Replicon cells used as positive control for the experiment, are stained with antibody against NS5A, using alexa-fluor 488-nm conjugated polyclonal IgG as a secondary antibody and with

antibody against albumin using PE-conjugated mouse anti-rabbit as secondary antibody.



Huh 7.5 cells

Figure 4.19: Double staining of Huh 7.5 cells: Huh 7.5 cells used as negative control for this experiment, do not stain positive with antibody against NS5A, using alexa-fluor 488-nm conjugated polyclonal IgG as a secondary antibody and with antibody against albumin using PE-conjugated mouse anti-rabbit as secondary antibody.



Fused cells (Day 3)

Figure 4.20: Double staining of the fused cells: Fused cells stained with antibody against NS5A, using alexa-fluor 488-nm conjugated polyclonal IgG as a secondary antibody and with antibody against albumin using PE-conjugated mouse anti-rabbit as secondary antibody.

Indirect immunofluorescence microscopy showed viral NS5A protein co-localised with albumin (produced by Huh7.5 cells), confirming replication in the fused cells.

4.3.4.3. Confocal immunofluorescence microscopy

Confocal microscopy was done to determine whether the NS5A staining of Huh 7.5s was due to protein within the cells or due to contamination from overlying monocytes. Confocal microscopy provided three dimensional images of the cells allowing a better appreciation of the site of the HCV proteins. Fused cells were stained at day 3 for NS5A using polyclonal sheep anti-NS5A as primary antibody while secondary antibody was alexa-Fluor 488 nm-conjugated polyclonal IgG (goat anti-sheep). Replicon cells were stained as positive controls and Huh 7.5s were taken as negative control. After that cells were stained for albumin (used as a hepatocyte marker) with polyclonal rabbit anti-albumin from DAKO, UK to differentiate Huh 7.5s from monocytes. The secondary antibody was alexa-fluor 565nm-conjugated mouse anti-rabbit from Invitrogen Molecular Probes UK. Replicon cells and Huh 7.5s stained positive for albumin. Fused cells stained positive for both albumin and NS5A, illustrated in figure 4.21.

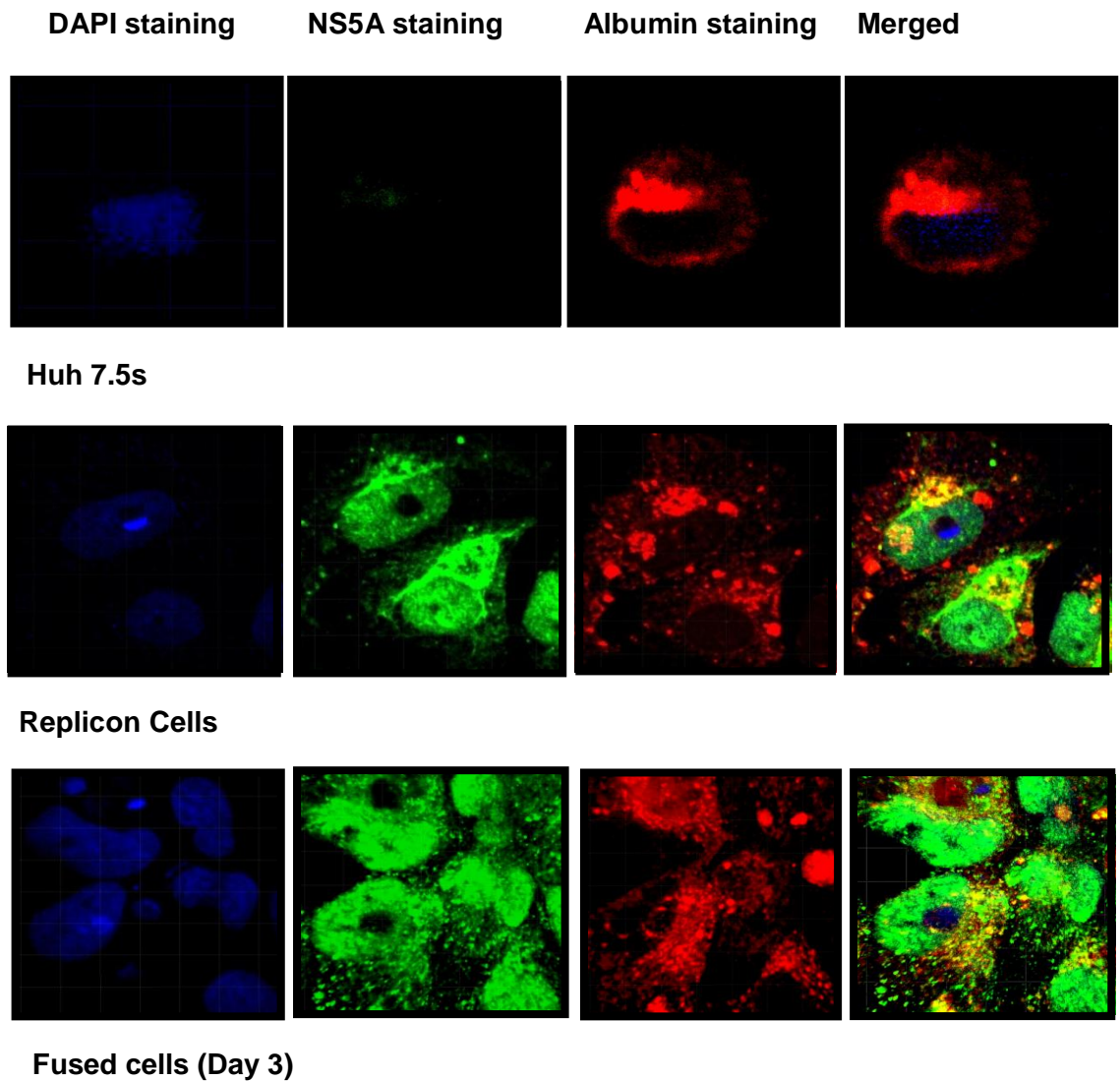


Figure 4.21: Confocal microscopy with dual staining: replicon cells, used as positive control for this experiment, are stained with antibody against NS5A; using alexa-fluor 488-nm conjugated polyclonal IgG as a secondary antibody and with antibody against albumin with alexa-fluor 565-nm conjugated mouse anti-rabbit as secondary antibody. Huh 7.5 cells, used as negative control for this experiment, do not stain positive with antibody against NS5A, using alexa-fluor 488-nm conjugated polyclonal IgG as a secondary antibody but stains positive with antibody against albumin with alexa-fluor 565-nm -conjugated mouse anti-rabbit as secondary antibody.

Fused cells stain with antibody against NS5A and with anti-albumin Ab showing double stain.

4.3.5. Manifestation of different types of fusions

Indirect immunofluorescence staining of fused cells showed that the cells may fuse in two different ways. In some cells 'intact monocytes' were linked to hepatocytes while some fused cells were seen as merged Huh 7.5 cells and monocytes. To examine the morphology of fused cells more closely, cells were fused after being stained with different fluoroprobes i.e.; DiO (wavelength similar to FITC) labelled Huh 7.5 cells and Dil (wavelength similar to PE) labelled monocytes were fused together under the previously determined optimal conditions and types of fusions were examined more closely under confocal immunofluorescence microscope (as discussed in Chapter 2: Materials and Methods). Confocal microscopy was used to see the position of monocytes relevant to Huh 7.5s in a fused cell. It was observed that monocytes fused with Huh 7.5 cells in two different ways. They either showed complete fusion with Huh 7.5 cells or fused partially.

Under the confocal immunofluorescence microscope complete fusion was observed as monocytes (IM) fused to Huh 7.5 cells and had irregular membrane and in this type of fusion 'spillage' of Dil dye was observed into Huh 7.5 cell cytoplasm. Partial fusion manifested itself as round monocytes (RM) either attached to Huh 7.5 cells or engulfed by Huh 7.5 cells with minimal 'spillage' of Dil dye into Huh 7.5 cell cytoplasm. Moreover, it was observed that monocytes showing more spillage of Dil dye into the cytoplasm

had much more irregular shapes than monocytes with lesser spill. It was also observed that the irregularity of monocytes coincided with the amount of Dil dye spilled into the cytoplasm of fused cell. Hence, there was a positive correlation between the amount of cytoplasmic spillage and irregularity of monocyte membrane which led to the hypothesis that the amount of extrusion of monocytic contents into the fused cells could be related to the extent of HCV replication into the Huh 7.5 cell fused with that monocyte. The mechanism by which monocytes might fuse in a particular fashion is not clear.

Figure 4.22, illustrates monocytes observed during the two types of fusion processes.

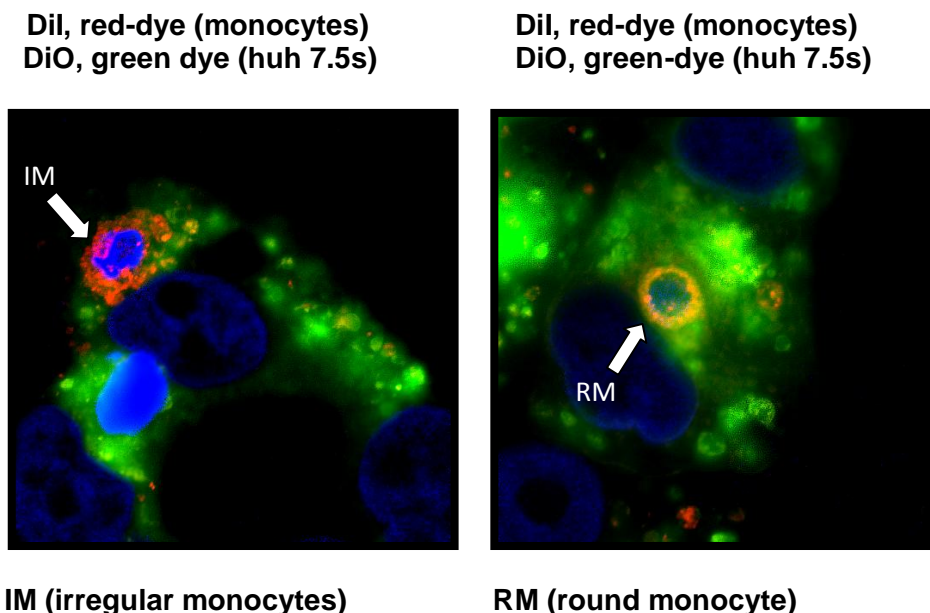


Figure 4.22: Types of monocytes in fusion: the two different type of monocytes (red stained with vibrant dye Dil) observed after fusion with Huh 7.5 cells (green stained with

vybrant dye DiO) are shown here with irregular monocyte (IM) on left hand side and round monocyte (RM) on right hand side.

4.3.5.1. Hepatitis C replication in different types of fusions

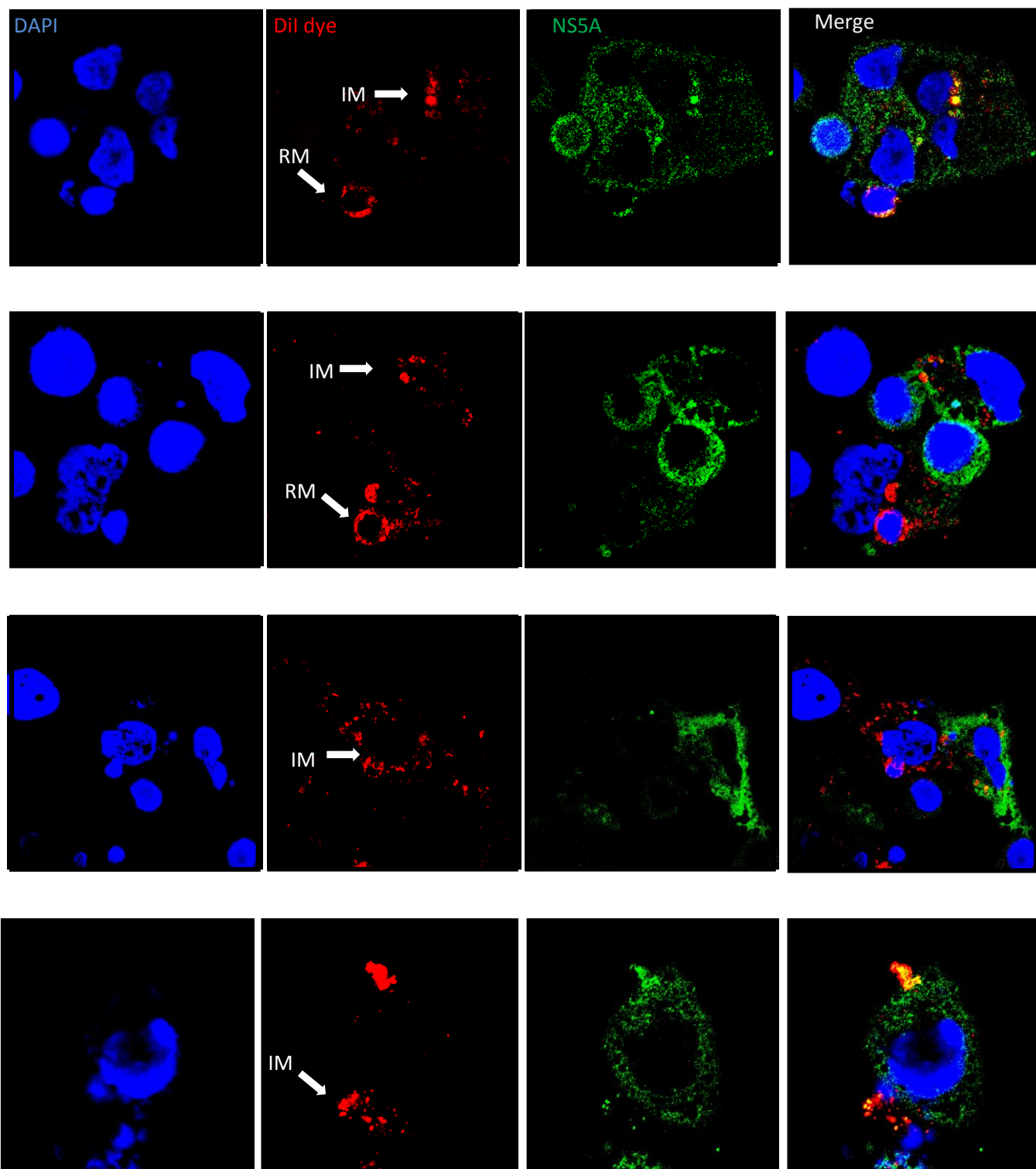
As discussed earlier it was speculated that two different fusion processes took place – one involved ‘extrusion’ of monocyte contents and the other involved the reconstitution of monocyte membrane and formation of an intact monocyte within the hepatocyte. It is possible that one of these fusion processes may be more permissive for HCV replication – we speculate that immortalisation of intact monocytes by fusion may allow HCV replication OR extrusion of monocyte contents may allow delivery of HCV into the replication permissive Huh 7.5 cells. To evaluate these possibilities and to determine whether to optimise the fusion process for delivery of intact or damaged monocytes we examined the level of HCV replication in fused cells of differing morphology.

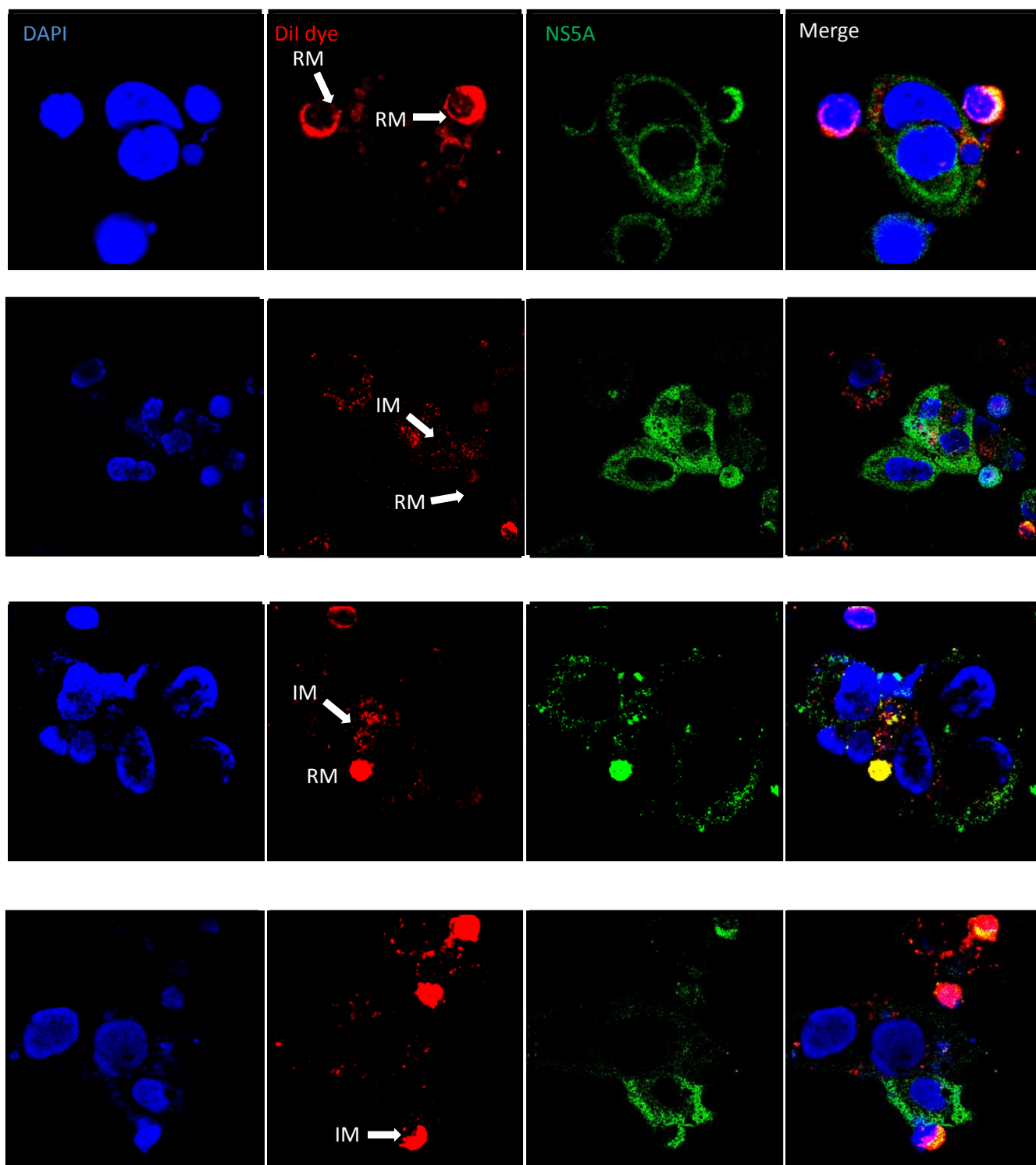
Dil dye labelled monocytes from two hepatitis C positive patients, fused with unlabelled Huh 7.5 cells and stained with primary antibody anti-HCV NS5A (secondary antibody is alexa-fluor 488) were used to study amount of HCV replication in the above mentioned two types of fusions. Different fields with NS5A signals in fused cells were randomly selected under confocal fluorescent microscope using 40x magnification, showing the two type of fusions i.e.; partial fusion (containing round monocytes-RM) and complete fusion (containing monocytes with irregular cell membrane- IM and spillage

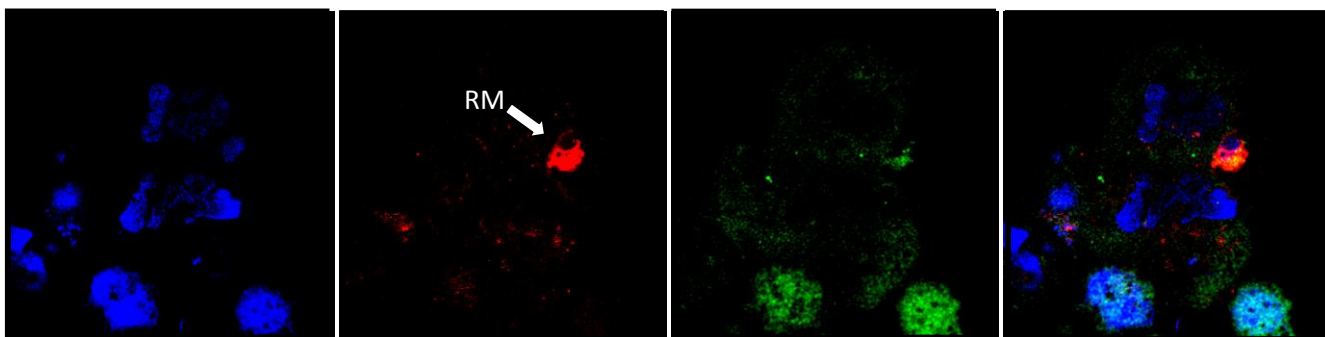
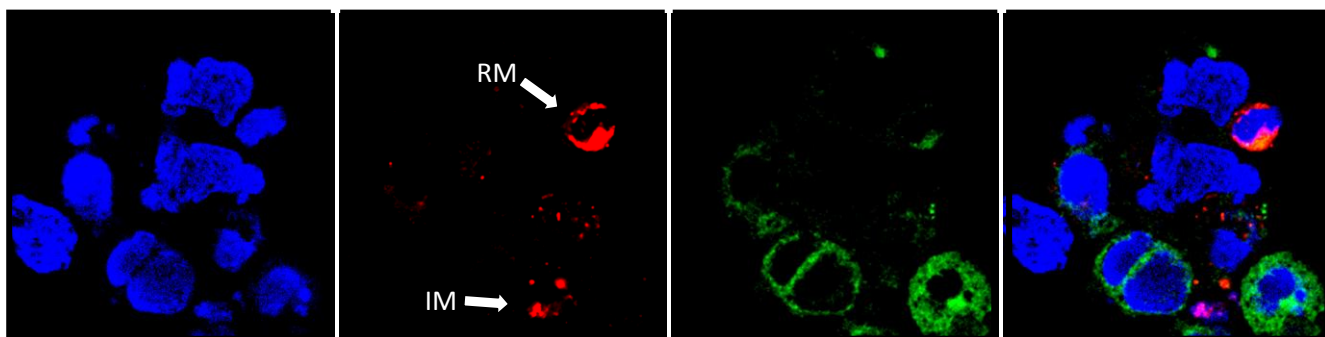
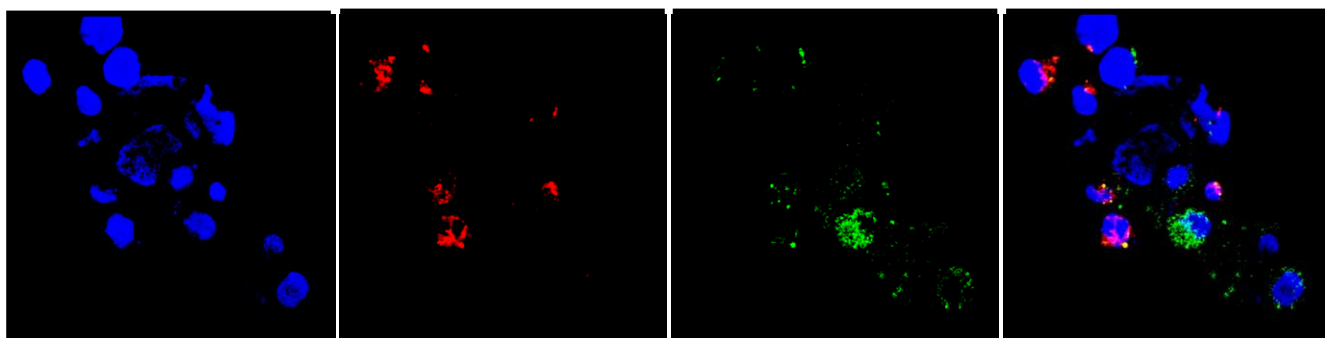
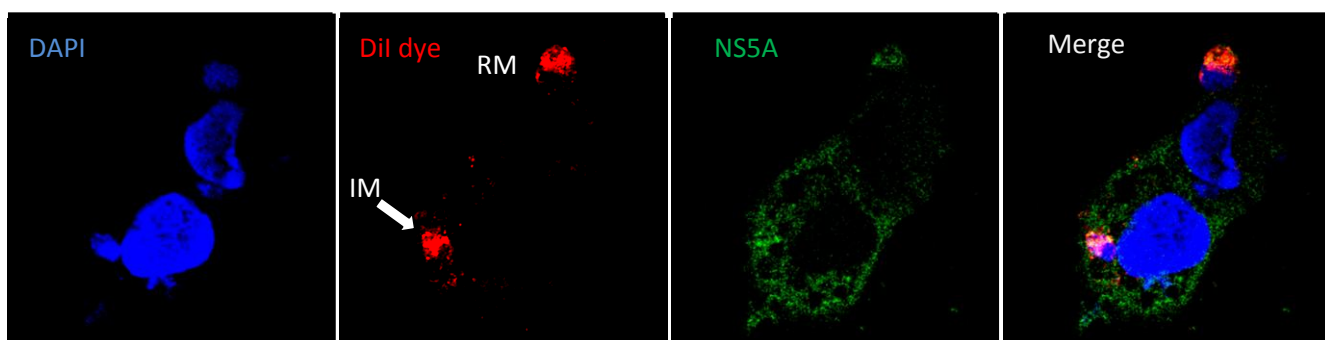
of cytoplasmic contents into the fused cell). Figure 4.23, shows the imaging slides of the two type of fused cells (containing either RM or IM) from two patients which were used to analyse HCV replication.

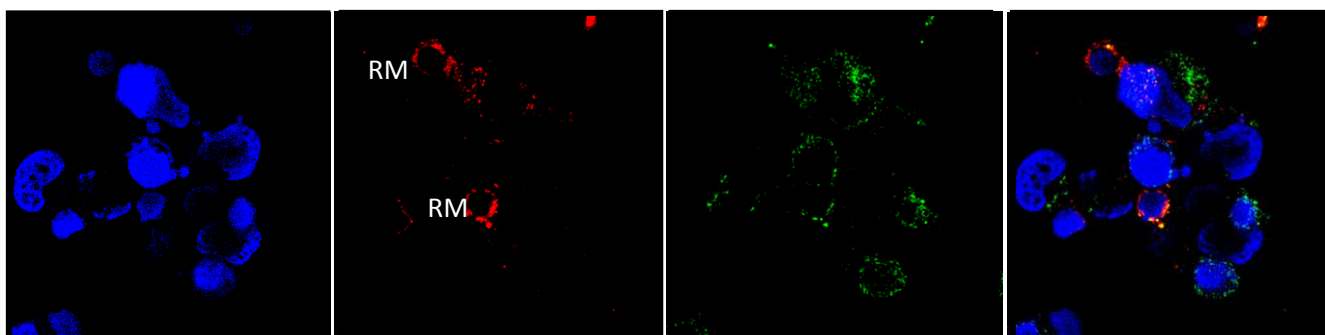
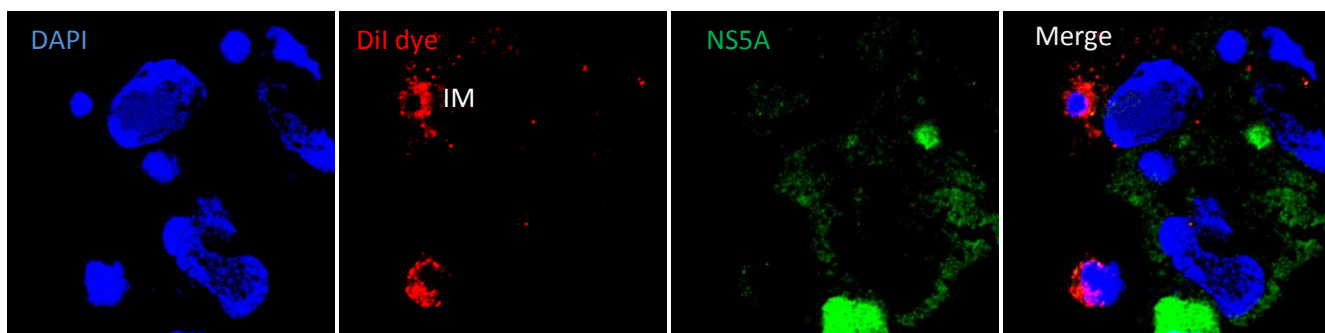
NS5A Staining of fused cells containing either round or irregularly
walled monocyte

Fused cells from patient A

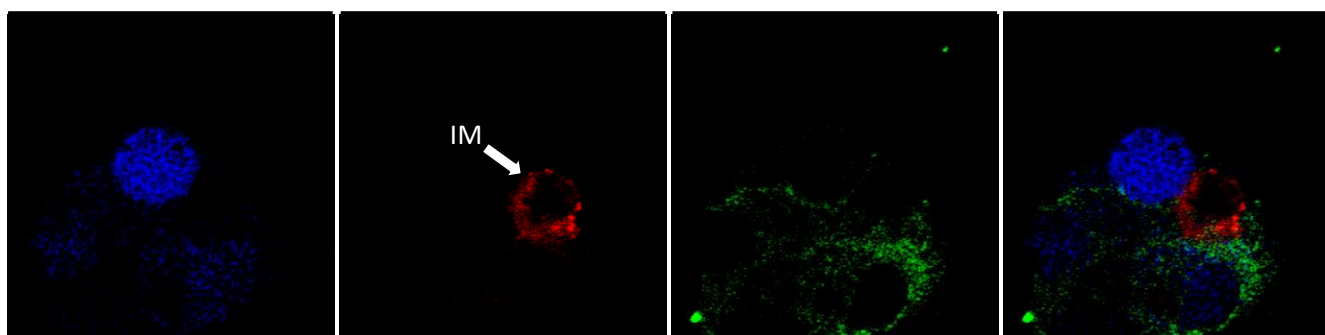
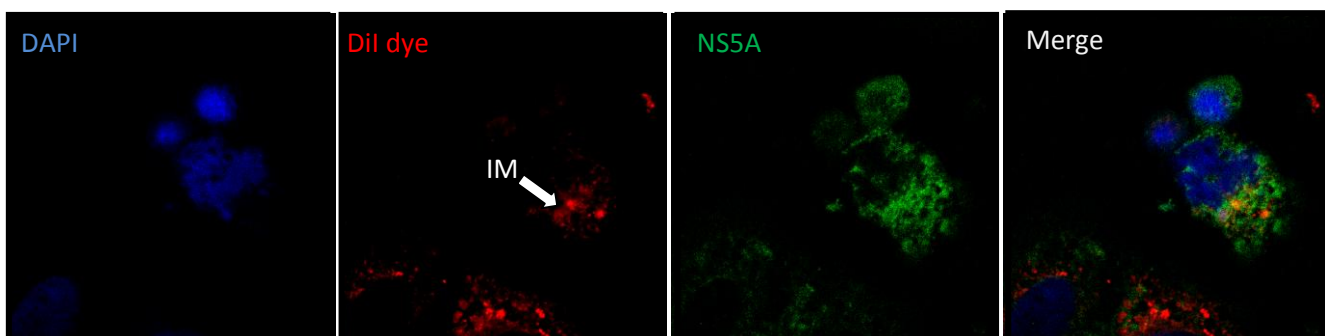


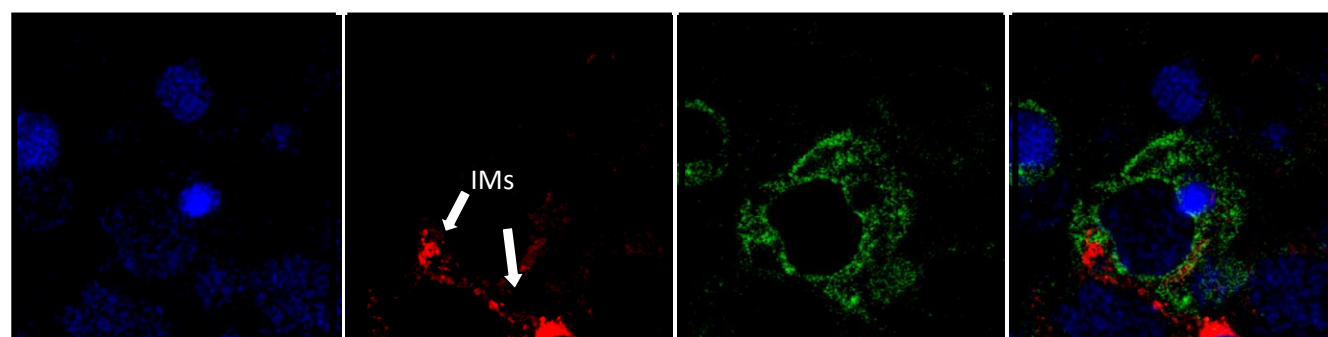
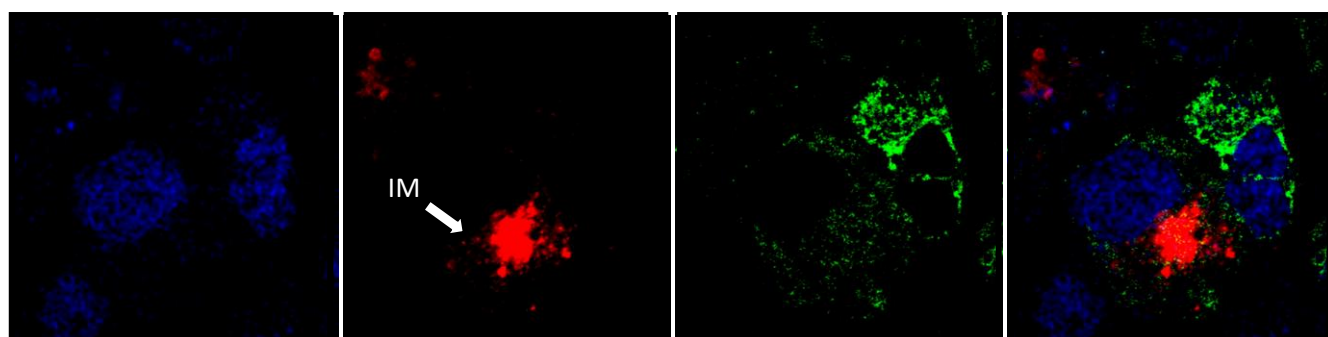
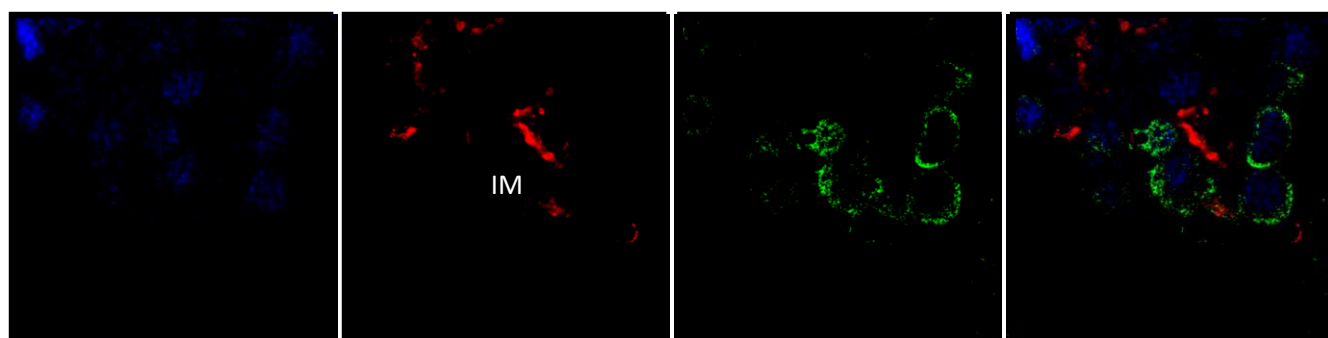
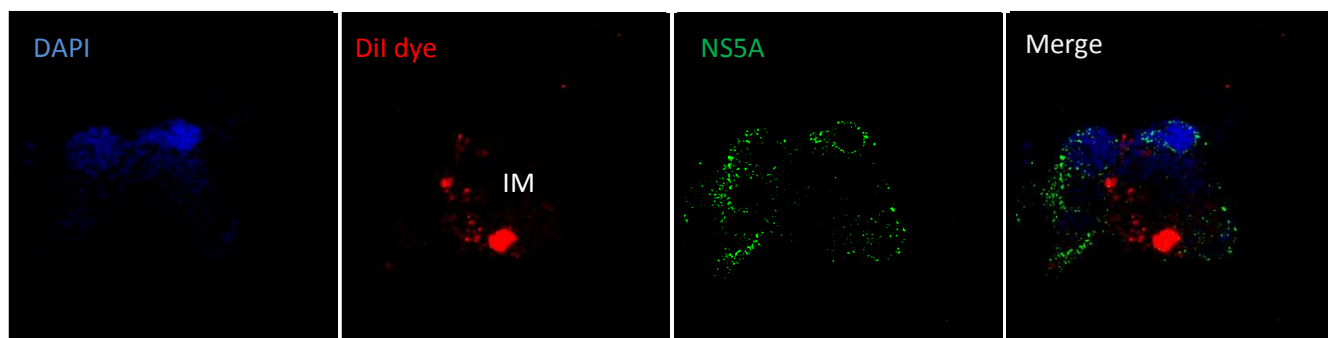


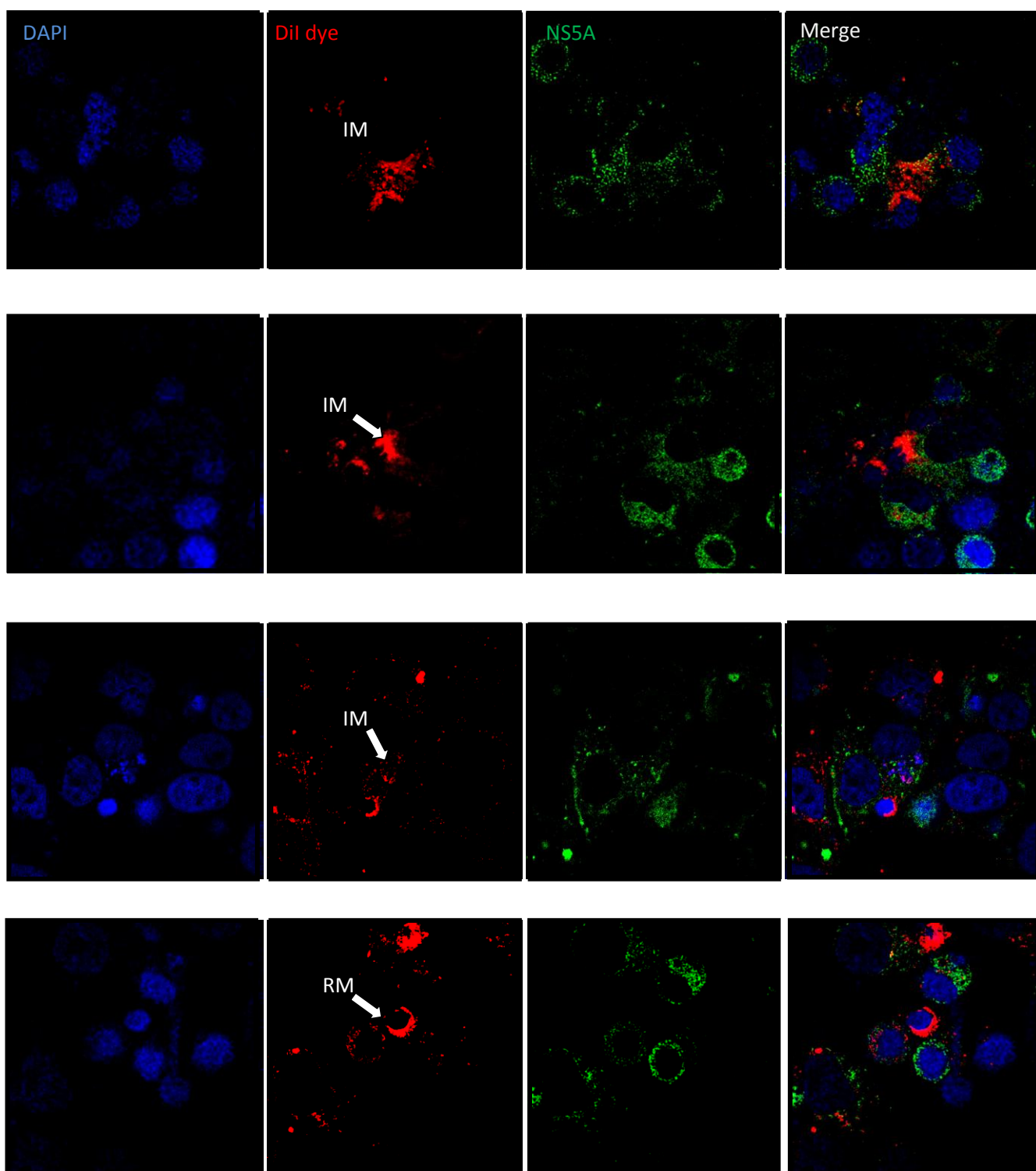


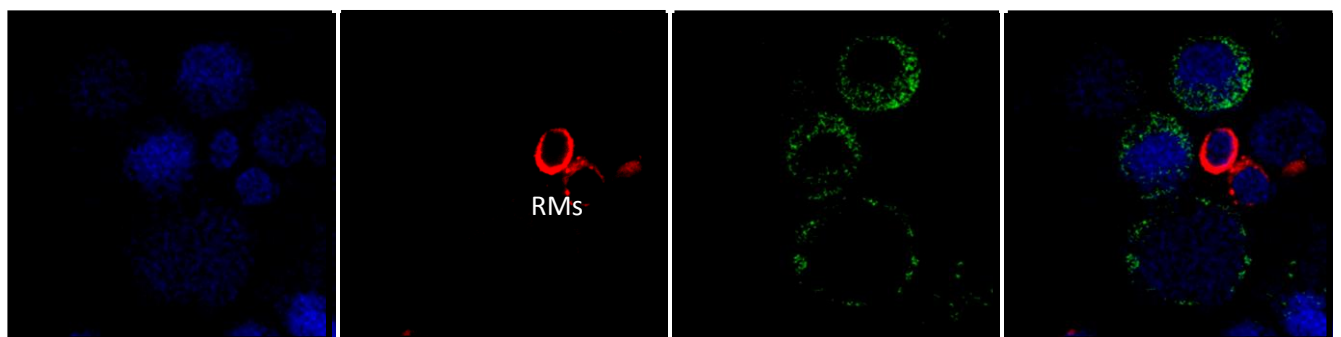
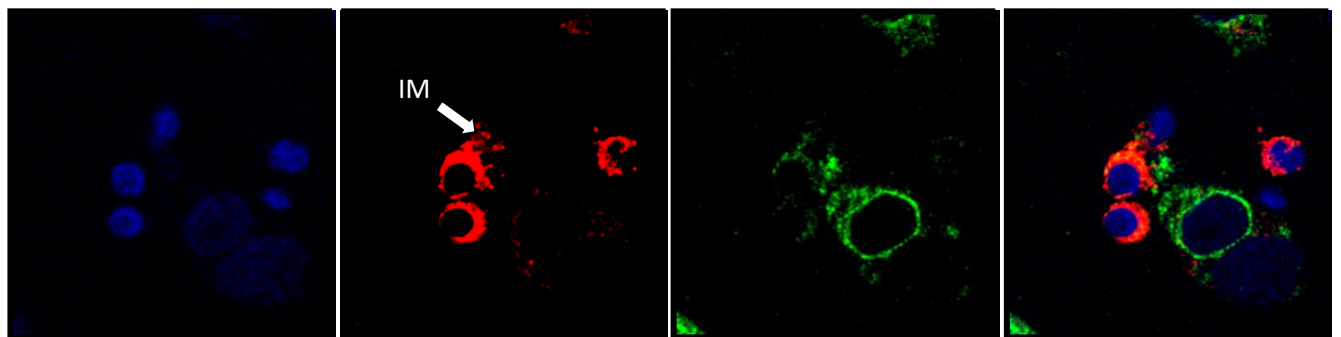
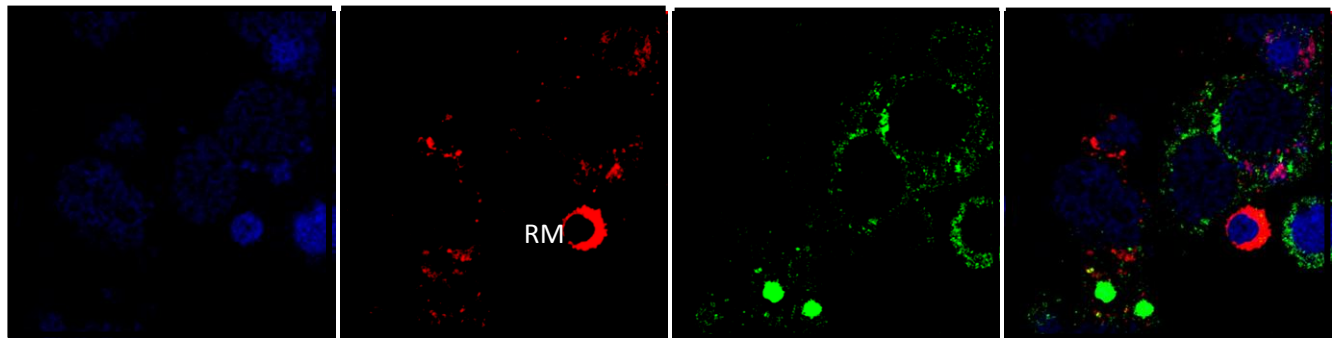
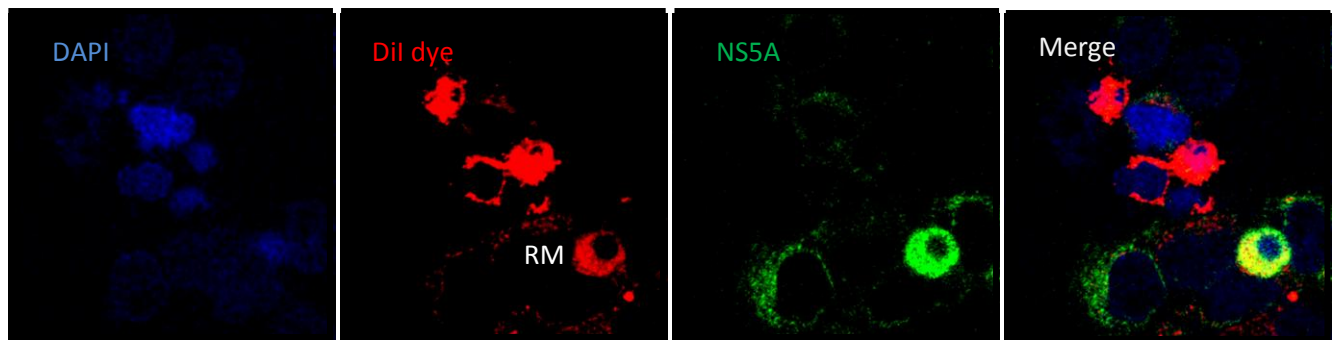


Fused cells from patient B









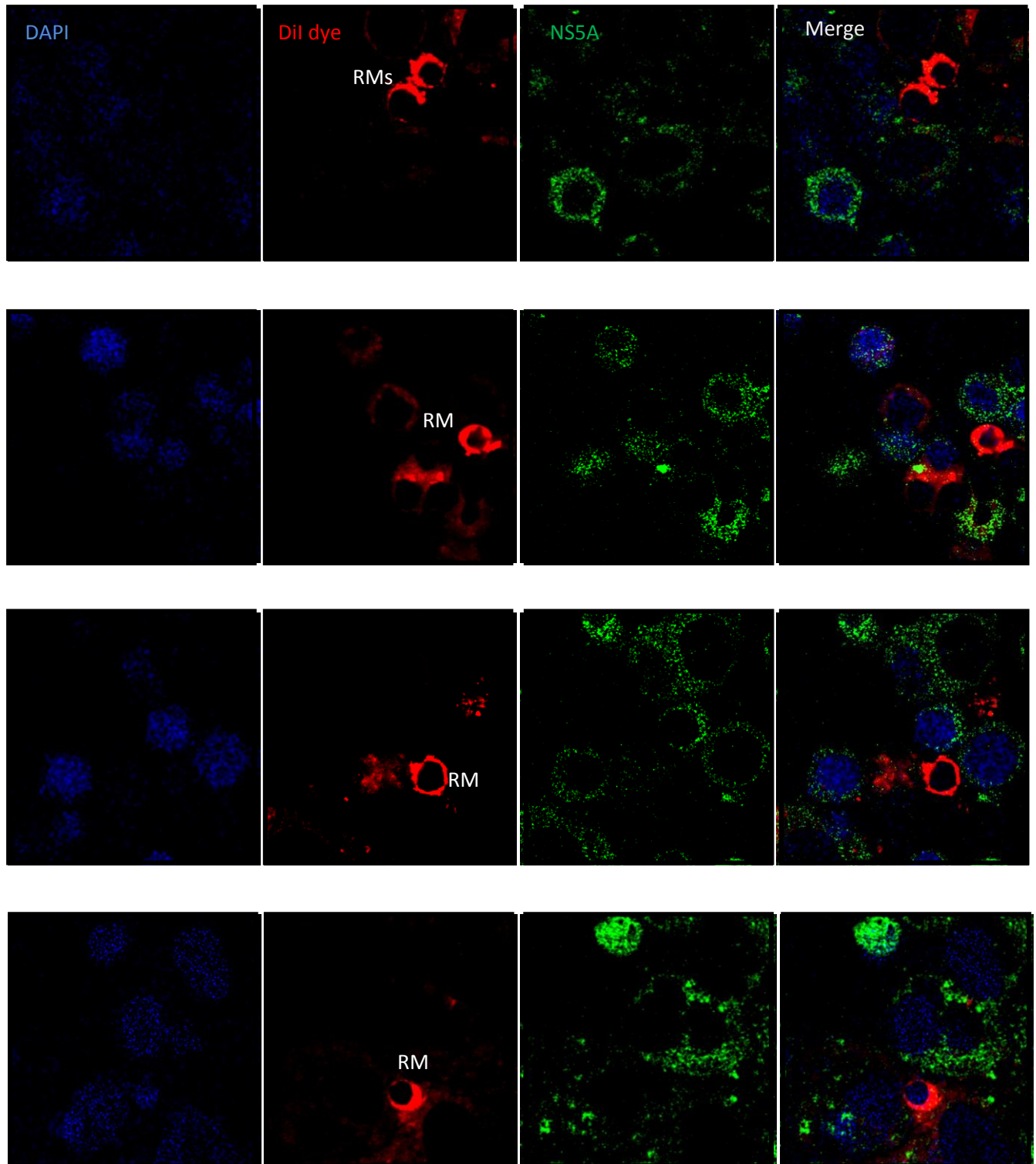


Figure 4.23: Anti-NS5A staining in irregular (IM) and round monocyte (RM) fusions: Monocytes stained with Dil dye (red). Anti-NS5A antibody staining is seen as green fluorescence (secondary antibody is alexa fluor 488). DAPI (blue), Dil stained monocytes (red) HCV NS5A staining of fused cells (Green).

4.3.5.2. Quantification of anti-NS5A staining

Monocytes from the two patients stained with Dil dye and fused with Huh 7.5 cells and then stained with anti-HCV NS5A antibody (secondary antibody-alexa fluor 488) were used to quantify amount of HCV replication in two different types of monocyte fusions. We speculated that the mode of fusion will effect the replication of HCV in the fused population. If fusion was complete it led to spillage of Dil dye from the monocyte into the cytoplasm of fused cell with or without the fusion of nucei and had irregular cell membrane under the microscope. Confocal microscopy was used to assess the position of monocytes in relation to the fused cell from different fields (detail discussed in above section) acquired after staining fused cells from two patients processed on different days. Image analysis and quantification was done as mentioned in materials and methods. Quantification was done in the selected population by chosing a region of interest (ROI). Area of selection, mean intensity of all pixels and integrated density (the sum of all pixels with in selected area) from selected population were noted. Tables 4.8 and 4.9 show areas, means and integrated densities from fused populations. Each table represents fused cells from a sinle patient, IM stands for irregular monocytic fusion while RM stands for round monocytic fusion.

Quantification of NS5A staining in two different types of fused cells from patient A:

No.	Area μm^2	Average Intensity	Integrated Intensity
IM-1	60092	45.03723	2706377
IM-2	40362	44.77967	1807397
IM-3	54500	84.69046	4615630
IM-4	16935	47.98264	812586
IM-5	19396	70.39096	1365303
IM-6	10357	52.35242	542214
IM-7	63730	68.59531	4371579
IM-8	59999	63.13229	3787874
IM-9	28867	24.72706	713796
IM-10	15643	32.06636	501614
RM-1	20495	45.7557	937763
RM-2	18565	22.80522	423379
RM-3	16223	25.41725	412344
RM-4	23511	34.89988	820531
RM-5	19288	30.9467	596900
RM-6	15026	25.4063	381755
RM-7	22948	14.95189	343116
RM-8	28017	15.67309	439113
RM-9	12335	33.40859	412095
RM-10	23507	14.14817	332581

Table 4.8: Patient A: Areas, means and integrated densities in the fused populations stained with anti-

NS5A: Quantification of HCV replication (NS5A staining)
 was done in the selected population by choosing a region of interest (ROI) and measuring area of selection, mean intensity of all pixels and integrated density in two types of fused cells i.e.; IM (irregular monocytes) and RM (round monocytes).

Quantification of NS5A staining in two different types of fused cells from patient B:

No.	Area μm^2	Average Intensity	Integrated Intensity
IM-1	29008	45.29578047	1313940
IM-2	76914	93.45224536	7187786
IM-3	56089	43.04915402	2414584
IM-4	21477	101.0451646	2170147
IM-5	36517	63.41824356	2315844
IM-6	31019	94.18830394	2921627
IM-7	35594	40.98151374	1458696
IM-8	30154	42.73598859	1288661
IM-9	33954	37.22459799	1263924
IM-10	16629	74.39755848	1237157
RM-1	46435	38.61233983	1792964
RM-2	36062	31.68382231	1142582
RM-3	39886	39.05565863	1557774
RM-4	63057	36.82760043	2322238
RM-5	32191	36.81252524	1185032
RM-6	52676	41.99500721	2212129

RM-7	34361	29.0168796	997049
RM-8	15123	27.99437942	423359
RM-9	24103	29.95668589	722046
RM-10	64442	34.40472052	2217109

Table 4.9: Patient B: Areas, means and integrated densities in the fused populations stained with anti-NS5A: Quantification of HCV replication (NS5A staining) was done in the selected population by choosing a region of interest (ROI) and measuring area of selection, mean intensity of all pixels and integrated density in two types of fused cells i.e.; IM (irregular monocytes in fused cells) and RM (round monocytes in fused cells).

The mean of NS5A staining area, average intensity and integrated intensity from fused cells with both IM and RM (from two patients) were used to compare HCV replication in these cells (shown in figure 4.24). Average intensity and integrated intensity were normalised to area of ROI.

Mean of area	
IM	36861.8 μm^2
RM	30412.55 μm^2

Mean of average intensity	
IM	58.47714754
RM	30.48862045

Mean of integrated intensity	
IM	2239836.8
RM	983592.95

Figure 4.24: Mean area, average intensity and integrated intensity of HCV NS5A staining compared between IMs and RMs from the two patients discussed above.

In order to compare the HCV replication and intensity of NS5A staining between the IM and RM, mean area of ROI, average intensity and integrated intensity were compared between the two.

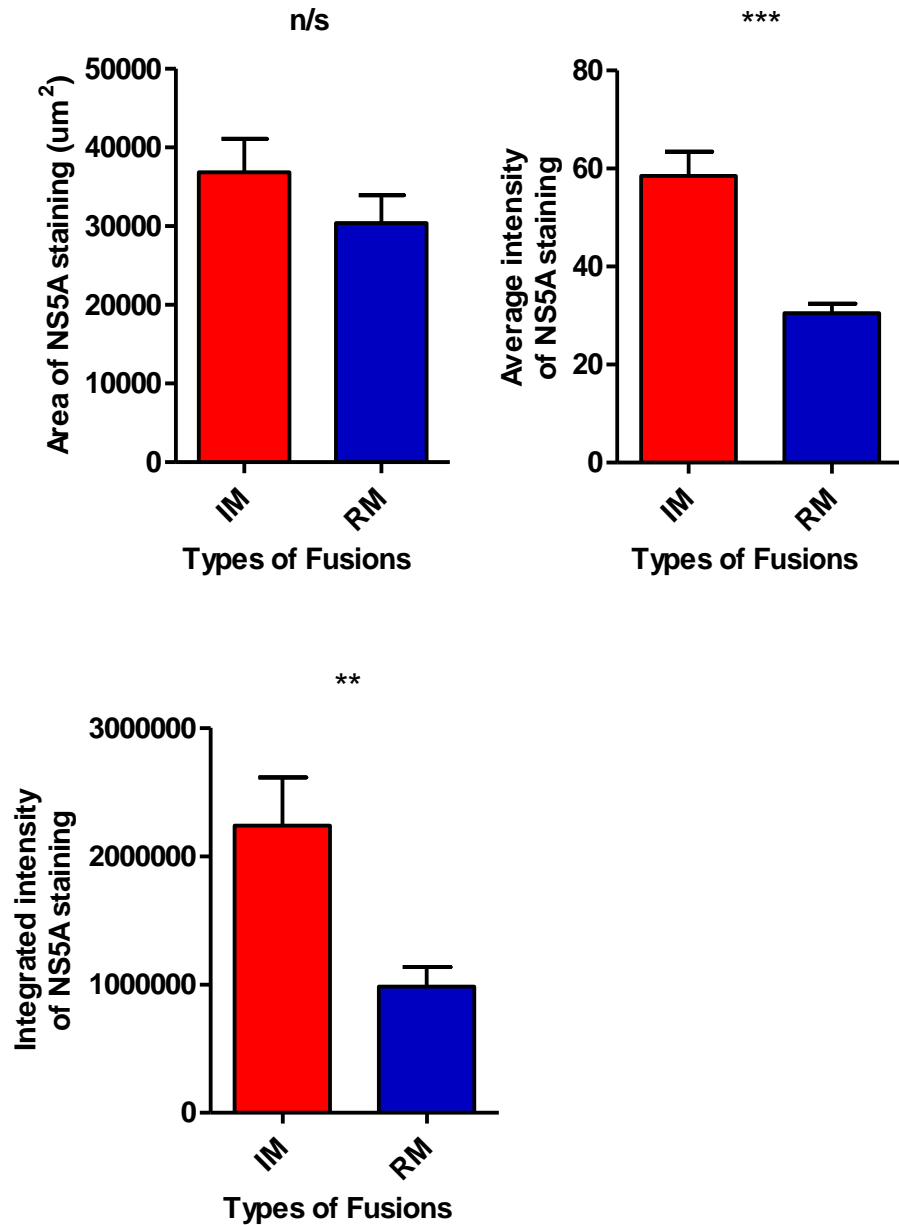


Figure 4.25: Quantification of hepatitis C replication from indirect immunofluorescent staining of NS5A in two types of fusions. A) Shows comparison between area of ROI (NS5A staining) measured in fused cells containing IM and RM. B) (p value = 0.0002) and C) (p value = 0.0023) compare intensity of NS5A staining between IM and RM. (IM-irregular monocyte fusion and RM-round monocyte fusion).

There is a significant difference in both average intensity and integrated intensity of NS5A stained area of IM and RM (area of ROI has no significant difference between IM and RM). These experiments suggest that different types of fusion may modify the replication of HCV in the fused cells and suggest that changing the fusion conditions to maximise the proportion of 'irregular' fusion may improve viral replication.

Time constraints prevented further development of this approach. Images for this experiment were taken with the help of Dr Ann Wheeler. In order to take images of 10 cells which had intact round monocytes and 10 irregular monocytes considerable time was spent. Finding these cells was considerably on the work intensive side as imaging needed 63x magnification to get a sufficiently detailed image to analyse (analysis was carried out along with Dr Ann Wheeler) which meant that only 1-2 cells could be taken per image. Thus this technique was not carried any further.

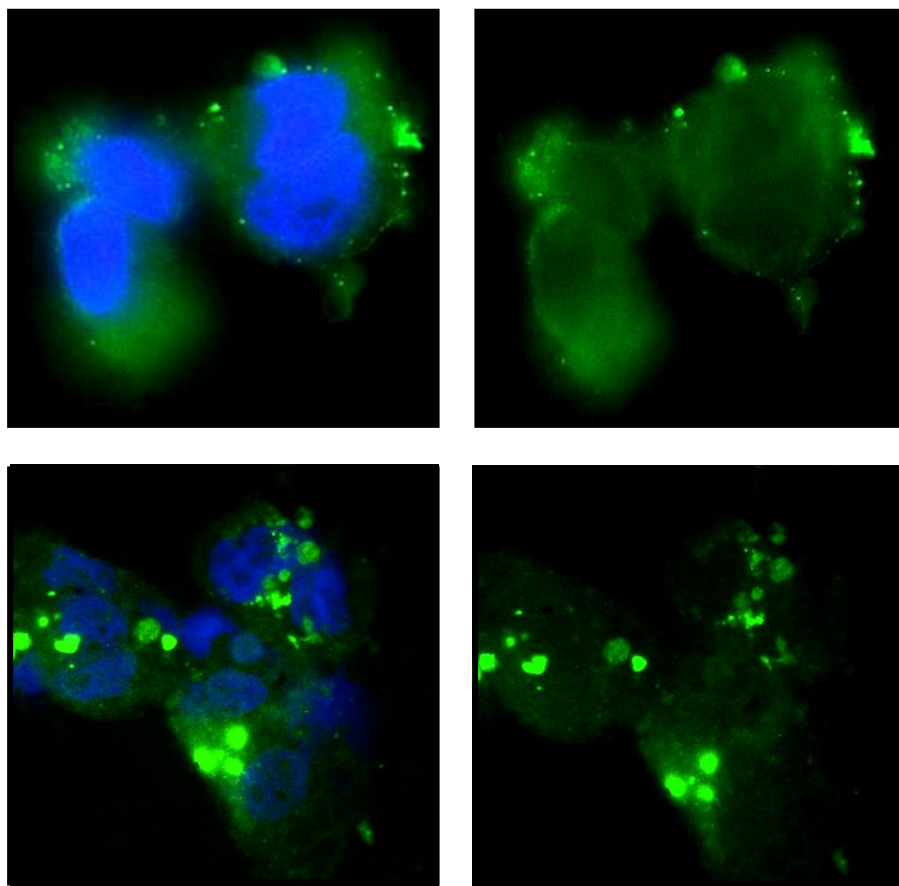
4.3.6. Monocytes fused with Huh 7.5 cells after incubating with patient's serum (capture transfer assay)

This section described a novel capture-fusion transfer technique using patient derived HCV to be 'captured' by monocyte and then used to infect hepatocytes using the cell fusion technique described above. This approach was used to extract patient derived HCV from hepatitis C infected serum so that eventually all genotypes could be studied without needing very large amounts of blood from HCV patients for the extraction of monocytes.

To determine whether we could infect cell cultured monocytes of healthy donors with HCV from serum of hepatitis C patient we incubated monocytes from uninfected volunteers with serum from a patient with chronic HCV. If we can transfer virus to monocytes then we speculate that we may be able to develop a 'capture-transfer' model of HCV replication. This will allow generation of infected cells using patient sera rather than patient derived monocytes. Monocytes from three healthy donors were incubated with serum of HCV patients for one day and fused with Huh 7.5s. Fused cells were then cultured for 3 days and stained for NS5A on day 3. This is illustrated in Figure 4.26.

Overlay of DAPI and NS5A

NS5A Staining



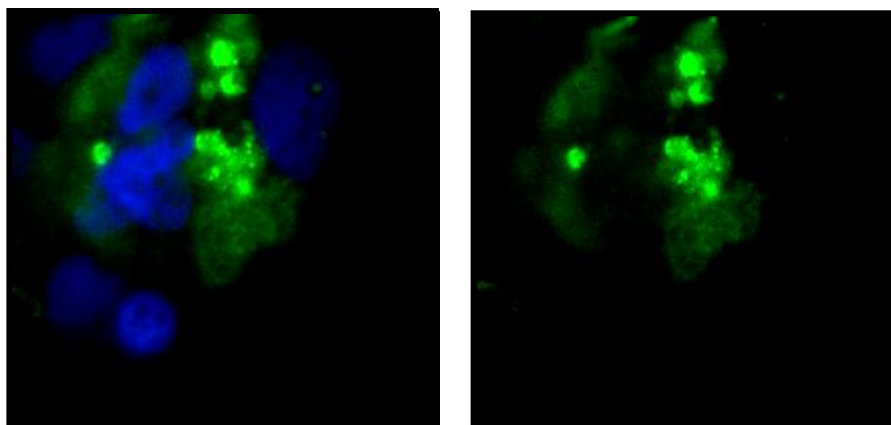


Figure 4.26: 'Capture' of HCV via monocytes and transfer to Huh 7.5 cells (n=3): Monocytes from healthy individual treated with serum of HCV patient and fused with Huh 7.5 cells and stained for NS5A to study HCV replication.

Time constraints prevented any further analysis of this approach. However, to extend the utility of this assay additional experiments were carried out by Dr Morven Cunningham who used the pre-stimulated monocytic cell line THP-1 instead of monocytes from healthy individuals to transfer HCV to Huh 7.5 cells using fusion technique and demonstrated successful replication for 5 days by real time qPCR technique and indirect immunofluorescence. Further experiments by Dr Morven Cunningham showed a genotypic pattern of response when treated with telaprevir which has little effect against genotype 3 but potently inhibits genotype 1 infection in clinical studies (Foster, Hezode et al. 2011, Jacobson, McHutchison et al. 2011, Zeuzem, Andreone et al. 2011). Similarly, alisporivir which shows response in genotype 2 and 3 infection compared to genotype 1 and 4 in clinical trials (Flisiak, Feinman et al. 2009) also showed a genotypic pattern of response when tried using this assay.

4.3.7. Fusion with SEAP reporter cell line

Recent publications suggest that an indicator cell line containing SEAP (secreted alkaline phosphatase) linked to a retention sequence via an NS3 cleavable bond can be used to identify replicating HCV (Iro, Witteveldt et al. 2009). Huh J20 cells harbouring this NS3/4A protease-based reporter assay kindly provided by Dr John McLaughlin, Glasgow. The aim was to determine whether this cell line would support replication in our assay and whether this detection system would allow easy detection of viral replication. We therefore used this liver cell line in our fusion assay. Monocytes from four patients (shown in figure 4.27) infected with HCV were used in this experiment. They were fused with the SEAP reporter cell line and incubated for 4 days. At days 1 and 4 medium was removed and used for the detection of alkaline phosphatase using a commercial kit (see Methods) as per the manufacturers recommendations. Placental alkaline phosphatase was used in 1:100 and 1:1000 dilutions as a positive control while plain DMEM was used as negative control. Luminescence is expressed as relative light units (RLU)

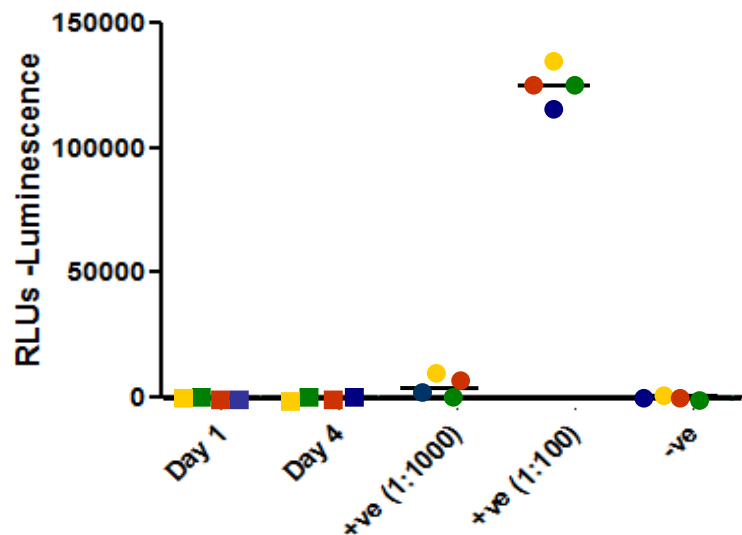


Figure 4.27: SEAP assay used to see whether it could detect HCV replication in fused monocytes and Huh J20 Cells: Amount of luminescence reflects the main principle of SEAP reporter cell line by secreting EGFP bound alkaline phosphatase in culture medium after being infected with hepatitis C virus because of cleavage at NS3/4A serine protease site by the virus. Positive control (placental alkaline phosphatase bound with fluorescent protein) is used in 1:100 and 1:1000 dilutions. Negative control was plain medium.

Two patients' monocytes were incubated with and without telaprevir at a concentration of 10µg/ml (14.7nM) before fusion with Huh J20 cells. Experimental controls were untreated monocytes (from similar patients) fused with Huh J20.

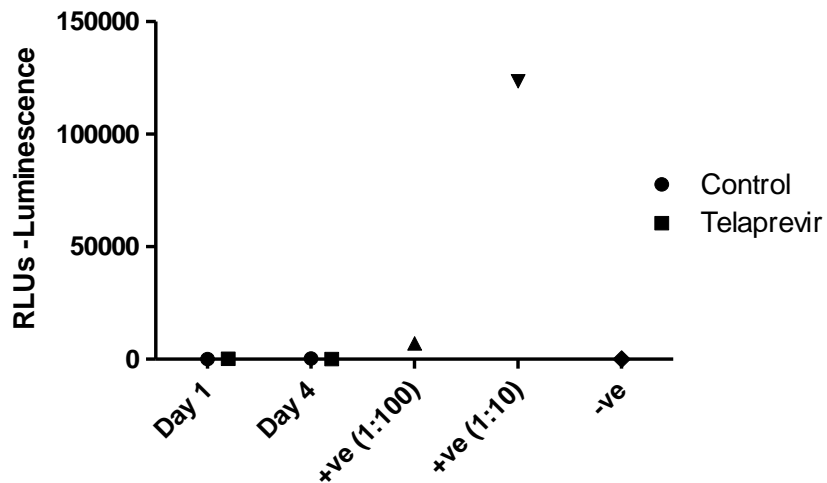


Figure 4.28: Patient A) Drug treatment of fused cells using SEAP assay to detect any change: Luminescence released by the fused cells which were treated with and without telaprevir along with the positive and negative controls is expressed as RLU.

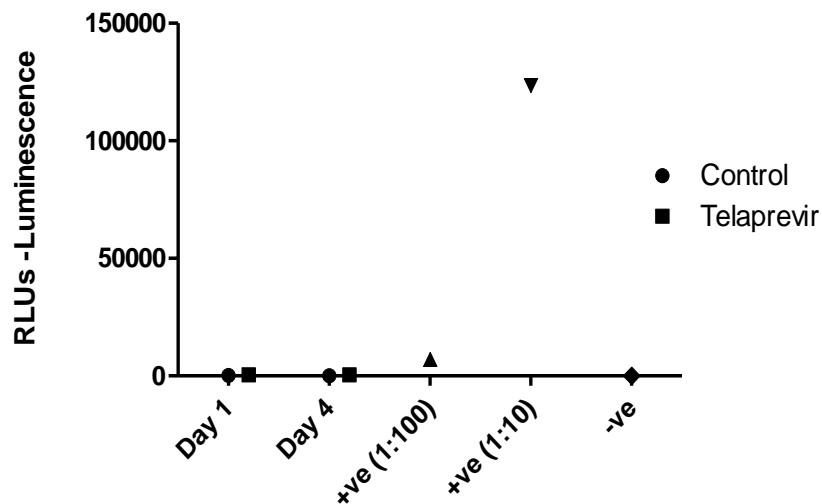


Figure 4.29: Patient B) Drug treatment of fused cells using SEAP assay to detect any change: Luminescence released by the fused cells which

were treated with and without telaprevir along with the positive and negative controls is expressed as RLU.

These experiments required further work as we did not include a positive control for the replicating hepatitis C virus which would cleave at NS3/4A site and release alkaline phosphatase in the culture medium. The positive control (placental alkaline phosphatase) used in the above experiments was provided in the SEAP kit and was already labelled with green fluorescence. It is not certain whether the conditions used in this experiment were optimised for replication of HCV in Huh J20 cells. Moreover, It is not clear from these negative experiments whether Huh J20 cells are permissive to viral replication or not. Furthermore, it is not known whether viral replication is taking place without protease mediated cleavage of recognition sequence in SEAP assay and subsequently stopping alkaline phosphatase release into supernatant. JFH-1 has shown to release alkaline phosphatase by recognising protease cleavage sequence in previous report by Pan et al. (Pan, Lee et al. 2009) which might implicate that SEAP assay is only positive with an aberrant HCV strain like JFH-1 which shows 20 fold higher transcription of HCV compared to replicon strains developed earlier and did not require adaptive mutation to sustain and replicate in infected cells.

4.3.8. Effect of PIV5-V protein on IFN induced pathway

The above studies revealed that in HCV fusion model the replication of HCV was marginal following fusion, although the amount of replicating virus was greater than that seen in isolated monocytes suggesting that there was some degree of enhancement in the fused cells. To increase the level of HCV

replication in fused cells different ways to enhance viral replication were tried. It was speculated that local IFN production may inhibit the replication of the fused cells and therefore a Huh7.5 cell line in which response to antiviral cytokines (type I, type II and type III interferons) was blocked by active deletion of signal transducer and activator of transcription 1 (STAT1) by the viral protein PIV5-V (parainfluenza virus 5-V protein) which degrades STAT1 was tried. In addition to blocking STAT1 it was planned to block other putative antiviral pathways that are induced by NF κ B.

To determine whether inhibiting the cellular response to interferon improved the replication of HCV in the fusion model we generated a cell line that lacks the ability to respond to Type 1, 2 and 3 IFNs. A stably transfected cell line containing PIV5 which is known to inhibit IFN signalling was generated for this purpose (see Methods) and I used this cell line in the fusion model. This cell line was obtained after transfecting Huh 7.5 cells by PIV-V.IRES.neo plasmid. The figure below shows the bands obtained by gel electrophoresis after restriction enzyme digestion of PIV-V.IRES.neo. To further confirm the integrity of the plasmid the full nucleotide sequence was verified by Gene Service UK (data not shown).

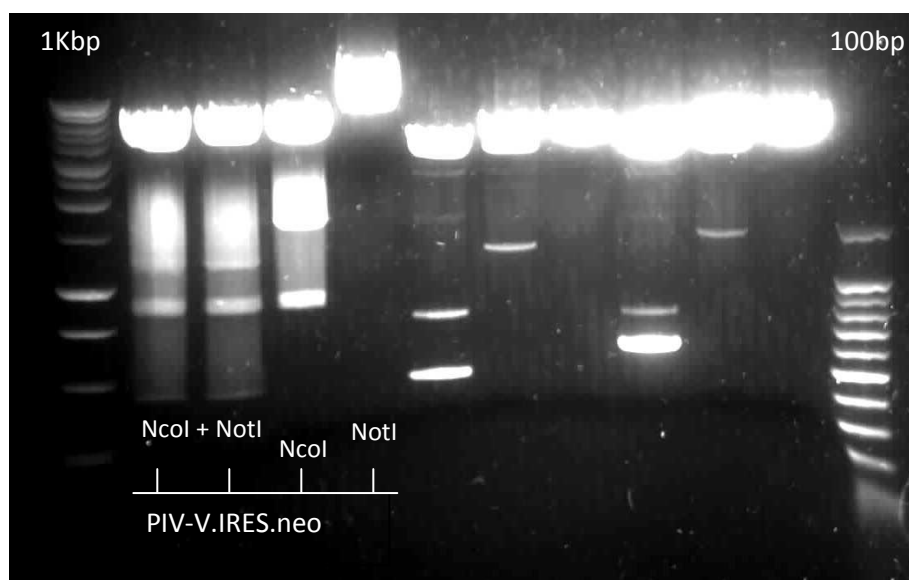


Figure 4.30: The bands obtained by the gel electrophoresis of the plasmid PIV-V.IRES.neo after restriction enzyme digestion at various points by the restriction endonucleases.

The predicted sizes of the plasmid digested with NcoI were 1631bp, 854bp and 5071bp. The predicted size of the fragment digested with NotI was 7556bp. Hence the plasmid restriction fragments are as predicted by the sequence.

After generating the clones of cells, i.e.; Huh 7.5 cells transfected with the PIV5-V containing plasmid, the ability of PIV5 to block the activation of IFN induced genes (ISGs) in Huh 7.5 cells was examined. The PIV5-V IRES.neo containing cells were treated with IFN (1000IU/ml) and tested for the expression of Interferon stimulated gene, MxA by real time qPCR after 24 hour incubation at 37°C and 5% CO₂. THP-1 cells and Huh 7.5 cells were used as positive controls for MxA production secondary to stimulation with IFN, illustrated in figure 4.30

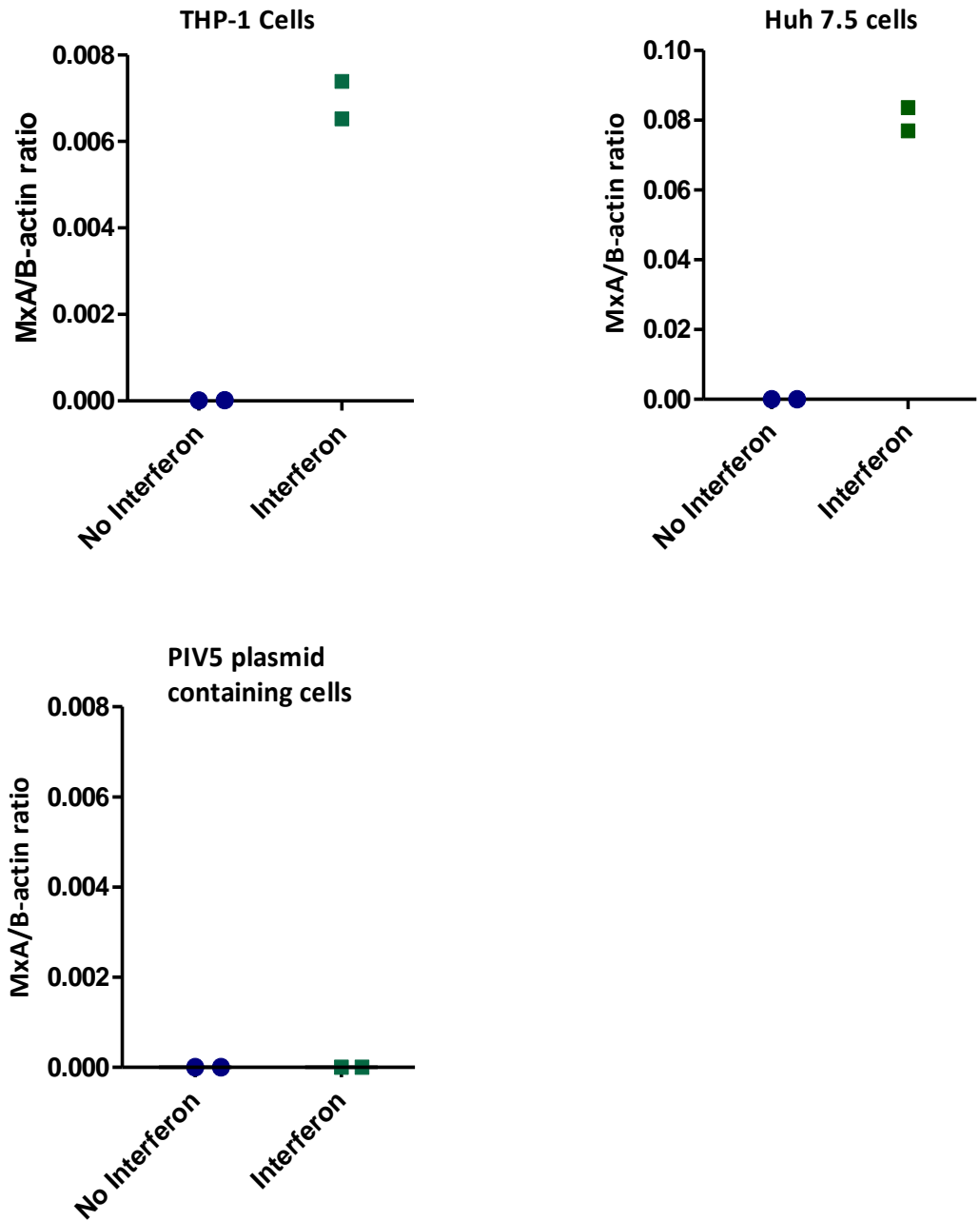


Figure 4.31: MxA expression in response to IFN stimulation: THP-1 cells, Huh 7.5 and PIV5 plasmid containing Huh 7.5 cells were treated with 1000 IU/ml IFN for 24 hours and expression of MxA was quantified by real time qPCR. The negative controls were not IFN treated.

The cell line containing PIV5 (PIV5 cells) does not show expression of MxA after stimulation with IFN while THP-1 and Huh 7.5 cells have high MxA expression on treating with IFN.

The next step was to fuse cells with no response to IFN with monocytes of hepatitis C patients and analyse HCV expression.

4.3.9. HCV expression in Huh 7.5 and PIV5 cells

Monocytes from patients with high viral load were fused with Huh 7.5 and PIV5 cells simultaneously and HCV expression and replication was assessed in both types of fused cells by real time qPCR and Immunostaining microscopy.

4.3.9.1. HCV expression through real time qPCR

Monocytes from seven different patients were fused with Huh 7.5 and PIV5 cells and HCV expression was assessed using real time qPCR at day 3 (n=4), day 5 (n=7) and day 7 (n=4). This is illustrated in figure 4.31 which shows comparison of HCV expression in both types of cells after fusion.

No	Date blood taken	Age	Genotype	Viral load	Fibrosis
1	20/08/2010	48	2a	5.98 log, 963648 IU	3
2	19/01/2011	30	3a	5.16 log, 142918 IU	2
3	18/02/2011	57	3a	5.19 Log, 153103 IU	4

4	07/02/2011	61	1a	6.46 log, 2866058 IU	3-4
5	01/03/2011	56	1	5.91 log, 815485 IU	1
6	01/03/2011	59	1a	5.94 log, 870964 IU	ND
7	20/04/2011	42	1	5.60 log, 396661 IU	2

Table 4.10: Details of the patients whose monocytes were fused with PIV5 positive Huh 7.5 cells. The table shows age, genotype and subtype (if available), fibrosis score (Ishak scoring) if liver biopsy was done, viral load done closest to the time of blood sampling from each patient. ND= not done.

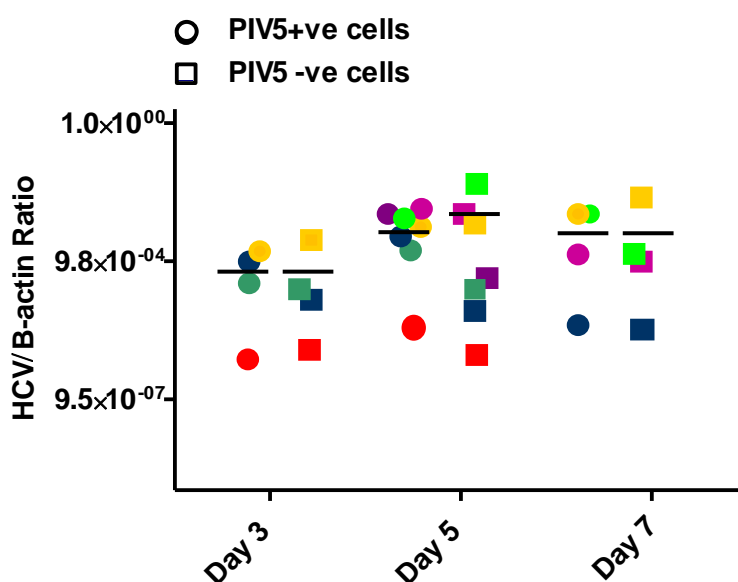
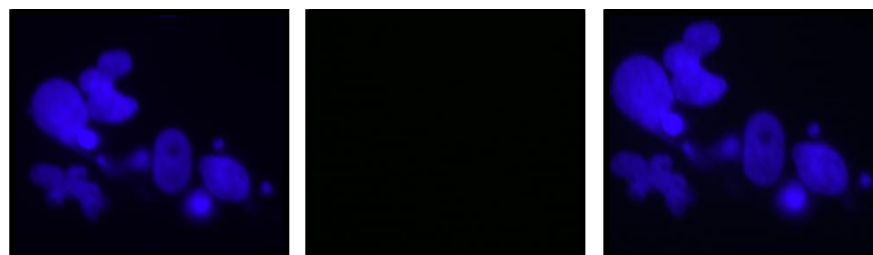


Figure 4.32: HCV expression in Huh 7.5 cells with and without PIV5 plasmid after fusion with patients' monocytes.

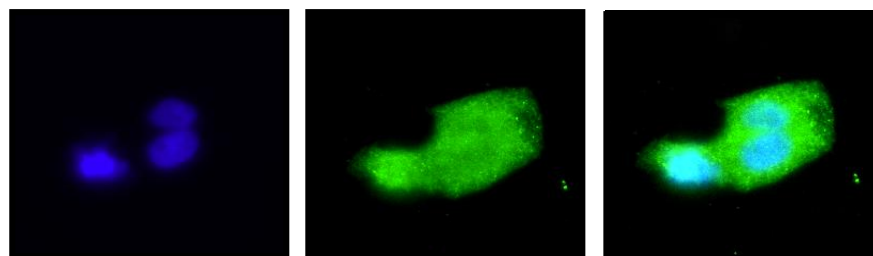
There was no significant difference in HCV expression and replication in both types of cells after fusion. P value was greater than 0.05 using Wilcoxon matched pair test.

4.3.9.2. Indirect immunofluorescence staining of PIV5 cells after fusion

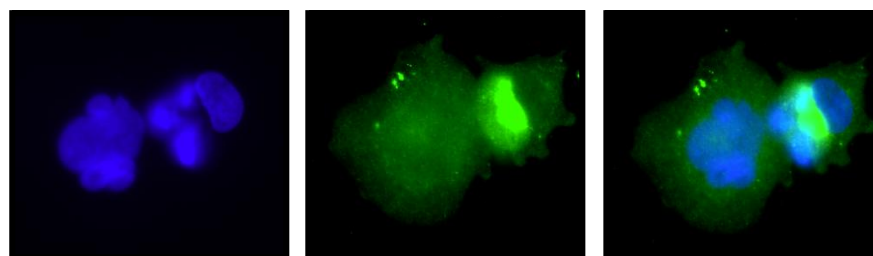
PIV5 cells were fused with three patients' monocytes and stained with antibody against NS5A.



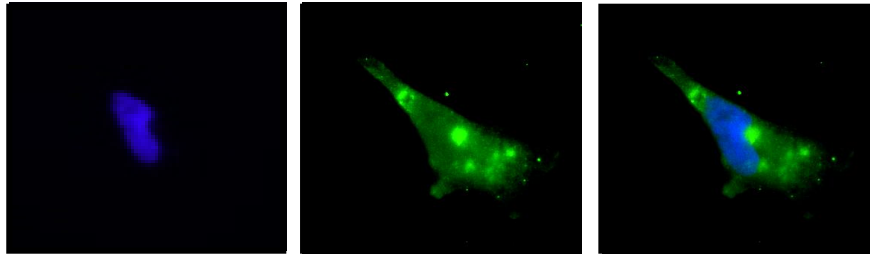
Isotype Control



PIV5 +ve Huh 7.5/ Monocytes fusion A



PIV5 +ve Huh 7.5/ Monocytes fusion B



PIV5 +ve Huh 7.5/ Monocytes fusion C

Figure 4.33: Indirect immunofluorescence staining of PIV5 containing Huh 7.5 cells after fusion with HCV patients' monocytes (n=3).

Taken together these data suggest that inhibiting IFN signal transduction does not augment HCV replication in this model. It is unclear whether this is because this replication model is insensitive to interferon or because interferon is not induced in this model system.

4.3.10. Patients used in indirect immunofluorescence microscopy experiments

The data of all patients whose monocytes were used for all the indirect immunofluorescence microscopy experiment are given below:

No	Age	Genotype	Viral load	Fibrosis	Experiment
1	57	2	6.65 log, 3650653 IU	ND	Fused cells
2	33	1b	6.89 log, 7714335 IU	ND	Fused cells
3	35	3a	6.78 log, 5968257 IU	5	Fused cells
4	59	4	5.94 log, 877674 IU	1	Fused cells (confocal microscopy)
5	58	1a	7.01 Log, 10280828 IU	3	Fused cells (confocal microscopy)
6	38	1b	7.15 log, 14287115 IU	3	Capture-fusion transfer
7	48	2a	5.98 log, 963648 IU	3	Capture-fusion transfer
8	30	3a	5.16 log, 142918 IU	2	Capture-fusion transfer
9	57	3a	5.19 Log, 153103 IU	4	PIV5 containing Fused cells
10	61	1a	6.46 log, 2866058 IU	3-4	PIV5 containing Fused cells
11	56	1	5.91 LOG, 815485 IU	1	PIV5 containing Fused cells

12	59	3b	5 log, 100000 IU	ND	IM vs RM fusion (confocal)
13	59	3b	5 log, 100000 IU	ND	IM vs RM fusion (confocal)

Table 4.11: Details of the patients whose HCV RNA expression was observed through indirect immunofluorescence microscopy after fusion: The table shows age, genotype and subtype (if available), fibrosis score (Ishak scoring) if liver biopsy was done, viral load done closest to the time of blood sampling from each patient. ND= not done.

4.3.11. Drug validation of HCV fusion model

A) Experiments with genotype 1 HCV containing fused cell

Monocytes from patients infected with genotype 1 strain of HCV were fused with Huh 7.5 cells. Equal numbers of cells were separated out with half kept as controls and other half were treated with telaprevir at a concentration of 10 µg/ml (14.7nM), known to inhibit the replication of Genotype 1 HCV. HCV expression was observed on days 3, 5 and 7 in both treated and untreated cells. Table 4.12 shows patients' data that were included in this study.

No	Date blood was taken	Age	Genotype	Viral load	Fibrosis	Fold Change On different days
1	20/04/2011	47	1a	6 log, 1000000 IU	2	Day 3: -1.01 Day 5: -188.8 Day 7: -1.88
2	27/04/2011	53	1a	7.20 Log,16017910 IU	2	Day 3: -8.80 Day 5: -3.07 Day 7: -26.2
3	04/05/2011	55	1	6 log, 1000000 IU	ND	Day 3: -98.1 Day 5: -277.3 Day 7: -22.7
4	11/05/2011	50	1b	NA	6	Day 3: -4.33 Day 5: -17.6 Day 7: -3.40

Table 4.12: Details of genotype 1 infected patients whose HCV RNA expression was observed with and without telaprevir treatment: The table shows age, genotype and subtype (if available), fibrosis score (Ishak scoring) if liver biopsy was done, viral load done closest to the time of blood sampling from each patient and fold change in HCV RNA in fused cells from different patients incubated with and without telaprevir. ND= not done, NA= not available.

Figure 4.33, 4.34 and 4.35 show the effect of telaprevir on HCV expression of fused cells from genotype 1 strain of HCV on day 3, 5 and 7 respectively and HCV expression was assessed by real time qPCR. Absolute quantification technique (using plasmid DNA standards - internal reference standards in form of plasmid containing target gene) was used to analyse HCV expression in treated and untreated samples. Absolute values were calculated for HCV from known standards. A known amount of plasmid was used to construct a calibration curve and then unknown samples identified from this and results were expressed in absolute terms as the number of copies of HCV RNA.

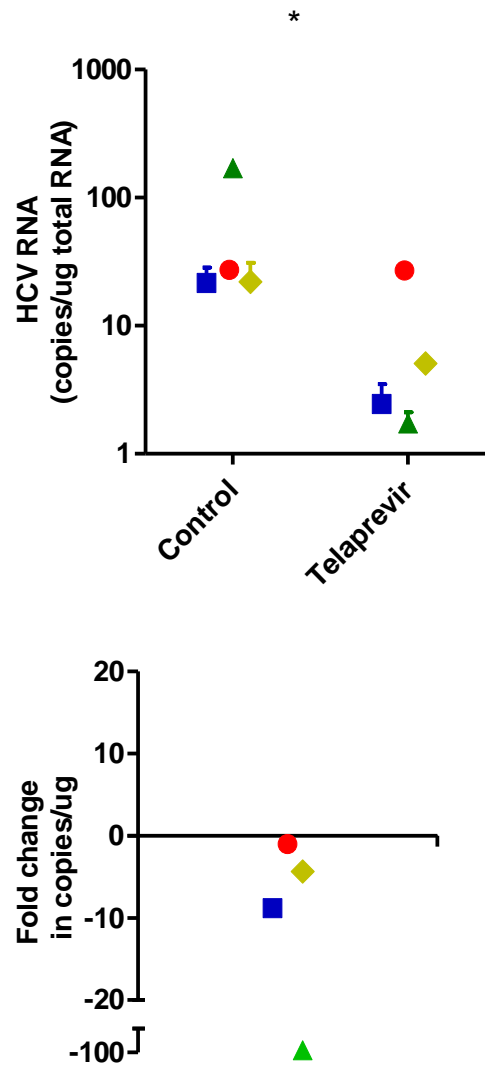


Figure 4.34: Effect of telaprevir on HCV expression of the fused cells from genotype 1 strain of HCV (from four patients) extracted on day 3: HCV expression was assessed by real time qPCR. Absolute quantification (using plasmid DNA standards) was used to analyse HCV expression in treated and untreated samples. Each point represents mean HCV expression from a single patient. *p = 0.0156 (using Wilcoxon matched pair test). Log10 scale is used for HCV RNA expression in top figure.

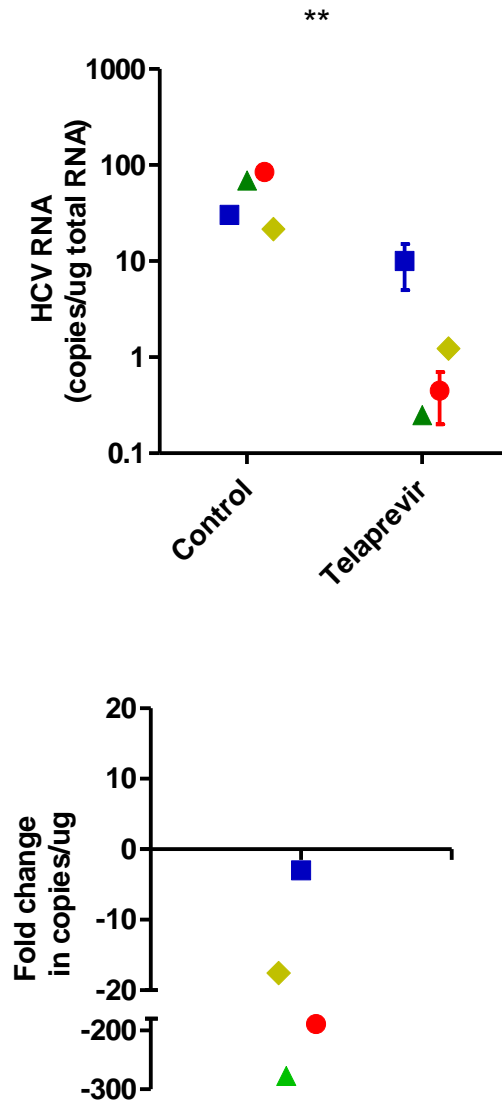


Figure 4.35: Effect of telaprevir on HCV expression of the fused cells from genotype 1 strain of HCV (from four patients) extracted on day 5: HCV expression was assessed by real time qPCR. Absolute quantification (using plasmid DNA standards) was used to analyse HCV expression in treated and untreated samples. Each point represents mean HCV expression from a single patient. **p = 0.0078 (using Wilcoxon matched pair test). Log₁₀ scale is used for HCV RNA expression in top figure.

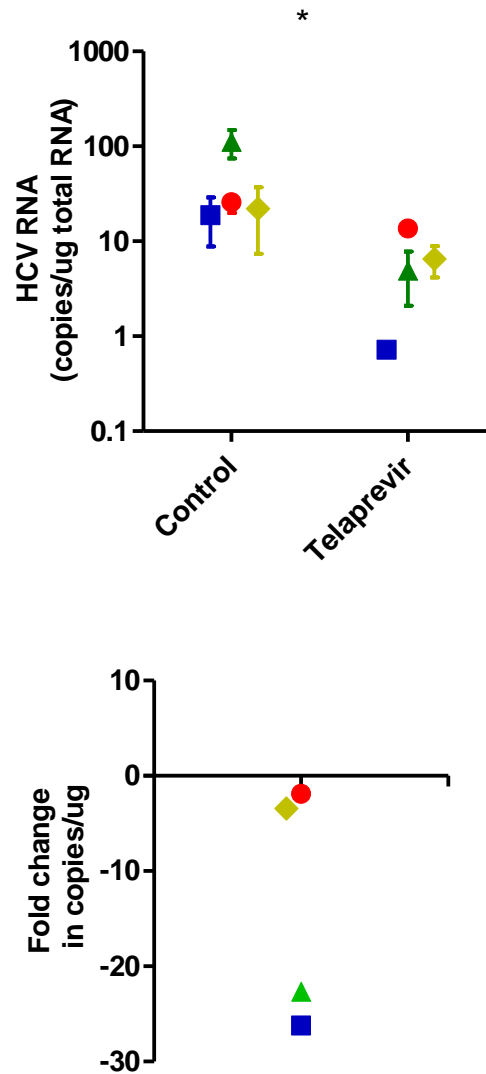


Figure 4.36: Effect of telaprevir on HCV expression of the fused cells from genotype 1 strain of HCV (from four patients) extracted on day 7: HCV expression was assessed by real-time qPCR. Absolute quantification (using DNA standards) was used to analyse HCV expression in treated and untreated samples. Each point represents mean HCV expression from a single patient. *p = 0.0156 (using Wilcoxon matched pair test). Log₁₀ scale is used for HCV RNA expression in top figure.

B) Experiments with genotype 3 HCV containing fused Cell

Clinical studies in patients infected with genotype 3 HCV show that telaprevir is ineffective in this genotype. To determine whether this can be detected in our assay we studied monocytes from patients infected with genotype 3 HCV. Monocytes from 4 patients were fused with Huh 7.5 cells. Equal numbers of cells were separated out with half kept as controls and the other half were treated with telaprevir. HCV expression was observed on days 3, 5 and 7 in both treated and untreated cells.

No	Date blood was taken	Age	Genotype	Viral load	Fibrosis	Fold Change On different days
1	18/05/2011	44	3a	6 log, 1000000 IU	5	Day 3: -2.29 Day 5: 1.66 Day 7: -1.84
2	18/05/2011	38	3b	6 log, 1000000 IU	2	Day 3: -1.64 Day 5: -4.30 Day 7: -1.79
3	25/05/2011	57	3a	6.23 log, 1699933 IU	ND	Day 3: 1.35 Day 5: 3.56 Day 7: 4.79
4	25/05/2011	59	3b	5 log, 100000 IU	ND	Day 3: 4.34 Day 5: 5.07 Day 7: 3.14

Table 4.13: Details of genotype 3 infected patients whose HCV RNA expression was observed with and without telaprevir treatment: The table shows age, genotype and subtype (if available), fibrosis score (Ishak scoring) if liver biopsy was done, viral load done closest to the time of blood sampling from each patient and fold change in HCV RNA in fused cells from different patients incubated with and without telaprevir. ND= not done.

Figure 4.36., 4.37 and 4.38 shows the effect of telaprevir on HCV expression of fused cells containing genotype 3 strain of HCV. HCV expression was assessed by real time qPCR. Absolute quantification technique (using plasmid DNA standards) was used to analyse results.

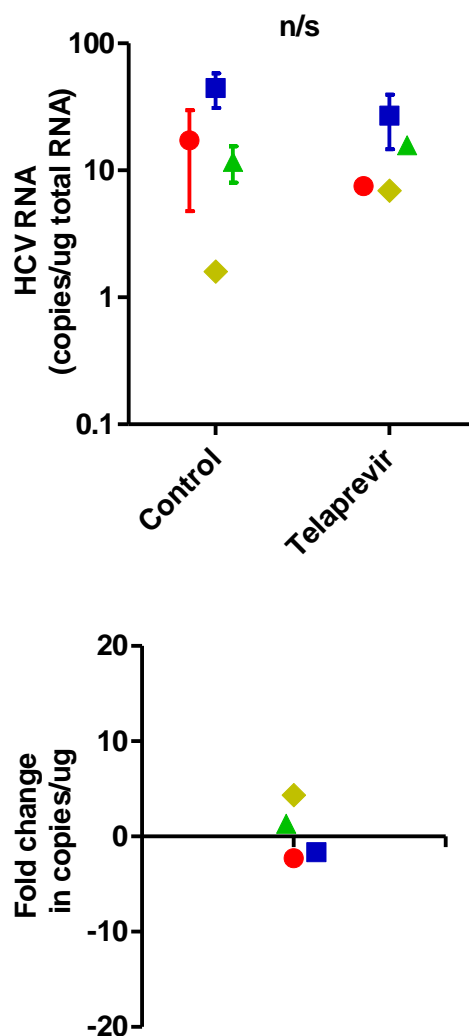


Figure 4.37: Effect of telaprevir on HCV expression of the fused cells from genotype 3 strain of HCV (from four patients) extracted on day 3: HCV expression was assessed by real time qPCR. Absolute quantification (using plasmid DNA standards) was used to analyse HCV expression in treated and untreated samples. Each point represents mean HCV expression from a single patient. $p > 0.05$ (using Wilcoxon matched pair test). Log10 scale is used for HCV RNA expression in top figure.

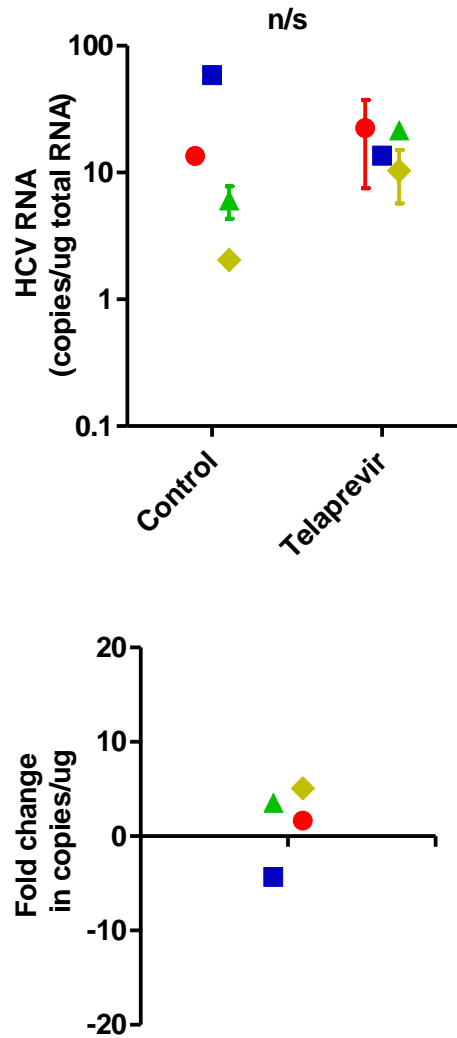


Figure 4.38: Effect of telaprevir on HCV expression of the fused cells from genotype 3 strain of HCV (from four patients) extracted on day 5: HCV expression was assessed by real time qPCR. Absolute quantification (using plasmid DNA standards) was used to analyse HCV expression in treated and untreated samples. Each point represents mean HCV expression from a single patient. $p>0.05$ (using Wilcoxon matched pair test). Log₁₀ scale is used for HCV RNA expression in top figure.

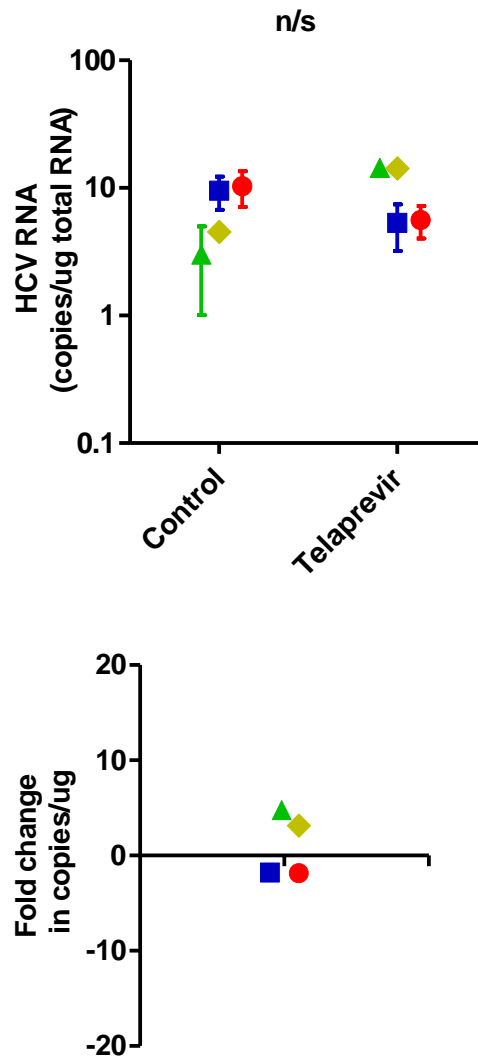


Figure 4.39: Effect of telaprevir on HCV expression of the fused cells from genotype 3 strain of HCV (from four patients) extracted on day 7: HCV expression was assessed by real time qPCR. Absolute quantification (using plasmid DNA standards) was used to analyse HCV expression in treated and untreated samples. Each point represents mean HCV expression from a single patient. $p > 0.05$ (using Wilcoxon matched pair test). Log10 scale is used for HCV RNA expression in top figure.

To compare the effect of telaprevir on genotype 1 and genotype 3 HCV all of the data containing C_T values from treated and untreated cells from patients with genotype 1 and 3 HCV were collated and plotted together. Figure 4.39 show the summary of all the data (note that this figure includes values from different times plotted all together i.e.; 3, 5 and 7 days from different experiments). In order to determine the reproducibility of the results reverse transcriptase step was done twice for each sample.

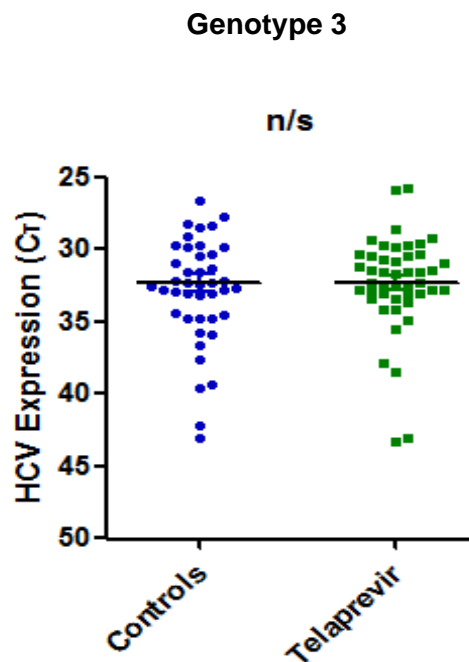
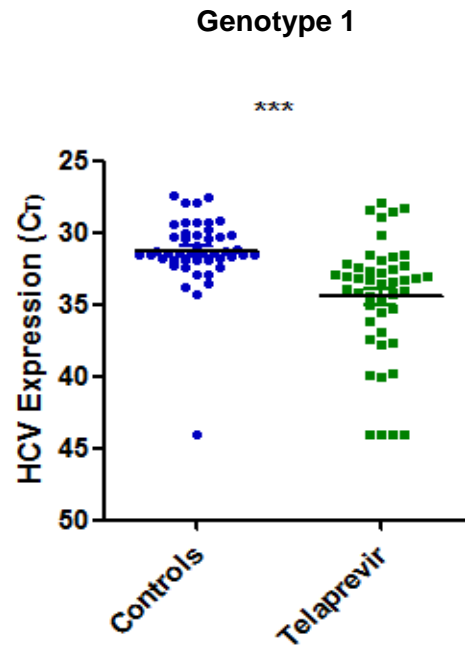


Figure 4.40: HCV expression shown as C_T values comparing differences between controls and telaprevir treated in both genotypes 1 ($p < 0.0001$ - matched pair test) and 3 (n/s): Each point in the graph shows a C_T value from the PCR of a different patient at days 3, 5 and 7 – RT (reverse transcription) done twice for each patient to look at reproducibility of the original result.

These data suggest that sensitivity to telaprevir in the fusion assay correlates with the clinical response. These initial drug sensitivity testing indicate that this assay may be useful to predict individual patient response to telaprevir.

Dr Morven Cunningham has now extended the work with antiviral drug testing of the fused cells from different genotypes and confirmed these preliminary findings.

4.4. Discussion

Accurate phenotyping of HCV before commencing on antiviral therapy is important for determining future response to conventional antiviral drug regimens. In recent years newer antiviral drug combinations have emerged. Drug combinations confer varying responses in variety of different HCV genotypes and subtypes (Zeuzem, Asselah et al. 2011). Cell fusion approach has been utilised by one previous group to study extrahepatic HCV replication (Frentzen, Hugging et al. 2011). We have applied this technique to allow an analysis of patient specific HCV strains.

Fusion data presented in this chapter shows that HCV expression can be detected in fused cells during the first few days of fusing monocytes of HCV patients with Huh 7.5 cells. The level of replication is low but can be inhibited by telaprevir in a genotype specific manner. However, the HCV signal in these fused cells is relatively low and our attempts to enhance the signal using inhibition of interferon signalling and modified detection techniques (SEAP release) were unsuccessful. Further steps need to be taken to

enhance current model and market for drug screening for 'difficult' genotypes (3, 4, 5, 6 etc).

We have set up an HCV fusion model based on approach recommended by Professor Malcolm Alison. Monocytes are one of the non-hepatic sites which are known to harbour replicating HCV (discussed in chapter 1 section 1.9.1). As discussed above serum derived HCV has not been propagated in hepatocytes. Moreover, HCV replicons show significant replication in Huh 7.5 cells compared to other cell lines. Hence, suggesting that Huh 7.5 cells are highly permissive to HCV replication but again they cannot capture HCV from patients' sera. It has been proposed that the block might be at the HCV entry step. Fusing monocytes with Huh 7.5 cells led to HCV replication within hybrids by bypassing this major hurdle. Furthermore, very low level of HCV RNA was detected within monocytes which were not fused and replication was much enhanced in fused cells for first few days. However, HCV RNA was significantly reduced in cultured fused cells after 1st week which could be due to over confluence of non-hybrid single Huh 7.5 cells due to selection advantage. This has hampered the formation of a cell line containing replicating HCV from individuals for long term drug sensitivity and resistance testing. In brief, our fusion technique has led to formation of a potent but short-term model which contains HCV obtained from patients. Although this model is cell type dependent it is robust and reproducible.

Real time quantitative PCR was used to detect HCV RNA. It is a sensitive assay which can detect very low level viral replication. This thesis relies heavily on data generated from real time qPCR reactions. There are many advantages of using real time qPCR but despite being described as the *gold*

standard, qPCR faces significant challenges because of variability of RNA templates, assay designs and protocols, as well as immense need for data normalization and conflicting data analysis. Appropriate standardization is required at every step of real time qPCR along with optimal assay design and reliable data normalization to obtain decent results. qPCR calls for optimisation in all channels of workflow that start with sample collection, sample stabilization and nucleic acid purification, and ends with amplification, detection and quantification of results. Apart from technical difficulties which include low yield, contamination and unspecific amplification, two major factors involved in PCR failure or poor results are; specificity of reaction and template secondary structure and factors influencing these two which are choice of enzyme, reaction conditions (annealing temperature, etc), primer design and template quality. Carefully choosing these factors is significant for better PCR quality and results. Due to exceedingly high sensitivity, well structured experimental design and a profound understanding of normalization techniques are essential for precise results. Variations during PCR can be incurred from different sources like assay design, PCR reagents, PCR equipments and human error. For example, lack of standardization of reverse transcription reaction can lead to variable results. Therefore, it is essential to measure intra- and inter-assay variation and also to minimize variability.

Despite the above mentioned challenges this technique can be very effective after considerable optimisation. The progress of qPCR reaction is effectively monitored as it occurs in real time. The amount of amplicon can be measured precisely at each cycle and the result is amazingly broad, 10^7 -fold dynamic

range, thus data analysis, including standard curve generation and copy number calculation, is performed easily. The amplification and detection is combined in a single tube eliminating the use of post-PCR manipulation. Post-PCR melt curve analysis is a simple way to check real time qPCR reaction for primer dimer artefacts and to ensure reaction specificity as different PCR products can be distinguished by their melt curve characteristics. It's easy to check PCR contamination by substituting negative control using water instead of a DNA template in one of the reactions. Moreover, this technique eliminates the need for post-amplification manipulation, significantly reducing the risk of contamination in subsequent experiments. Moreover, a well constructed experiment and introducing proper controls can make real time qPCR one of the most sensitive, efficient and reproducible methods of calculating gene expression. In our experiments we used appropriate controls at various stages to monitor the reliability of this technique. Considerable steps were taken to eliminate the issues with contaminations. Appropriate primers were used during the final amplification stage to achieve correct amplicons.

We have used immunofluorescence (IF) microscopy to confirm presence of HCV viral protein NS5A in the fused cells which signifies HCV replication. From both PCR and IF microscopy results it has been confirmed that replication is taking place in fused cells. qPCR results show that replication in fused monocytes and Huh 7.5s is significantly enhanced compared to monocytes alone. The work in this thesis could not demonstrate release of HCV virion in supernatant. However, later experiments by Dr Cunningham

using a modification of this assay with a new monocyte cell line (THP-1 cells) have shown virus release suggesting that the virus is undergoing complete replication.

In order to amplify replication of HCV in fused cells different techniques were tried; initially to enhance fusion between monocytes and Huh 7.5s and by modifying fusion process and later by interfering with IFN induced signalling and inhibiting response to IFN. It was observed that inhibiting IFN induced response in Huh 7.5 cells did not increase replication in fused cells when compared with regular Huh 7.5 cells. One of the reasons for this blunted response to inhibited IFN signalling may be that Huh 7.5 cells have defected RIG-1 signalling which inhibits the production of IFN, thus Huh 7.5 cells are already deficient in IFN and hence the reason why they are so permissive to HCV infection (discussed in chapter 1 section 1.7.3.1). Our data may confirm the previous reports that Huh 7.5 cells are defected in RIG-1 signalling which makes them ideal for HCV replication.

In this chapter I have described preliminary experiments done to set up a novel 'capture-fusion' assay in which patient derived HCV is 'captured' by monocytes and then transferred to hepatocytes using cell fusion. This work was extended further by Dr Cunningham who showed that this technique allows analysis of patient-derived virus and drug sensitivity and treatment response can be predicted for a wide spectrum of clinical isolates. It has been postulated by Revie et al. that monocytes/macrophages might up-regulate certain factors which could modify HCV and promote its infection in various other cell line or select variants which could have selection advantage (discussed in chapter 1 section 1.9.1). Dr Cunningham showed

that pre-stimulation of THP-1 cell line by empirically chosen cytokine profile led to success of capture transfer assay and without this prior stimulation HCV could not be detected in Huh 7.5 cells after incubating monocytes from buffy coats with sera of patients and then fusing with Huh 7.5s. My results with unstimulated and uninfected monocytes from buffy coats showed HCV transfer in fused cells after incubating these monocytes with patient's sera. This observation does not coincide with later experiments by Dr Cunningham using unstimulated THP-1 cells which showed lack of HCV transfer in fused cells. One explanation could be that monocytes/macrophages select out HCV variants from patient's sera or they might up-regulate various factors in Huh 7.5s that increase HCV transfer in their fused cells. Moreover, as cytokine expressions in monocytes and THP-1 cells vary following stimulation with LPS it is possible that varying results obtained using THP-1 cells in capture-fusion transfer technique may be due to this difference (Schildberger, Rossmanith et al. 2013).

Telaprevir is a protease inhibitor which inhibits the hepatitis C viral enzyme NS3 4A serine protease. It establishes covalent but reversible bond with the protease catalytic site in order to inhibit the action of NS3 4A serine protease. It is indicated for treatment of genotype 1 HCV infection. We chose to use telaprevir for drug validation experiments because its potent, rapidly acting and its response varies in different genotypes. Telaprevir efficacy in the treatment of genotype 1 hepatitis C infection has been demonstrated in numerous clinical trials and after instituting it in the treatment of genotype 1 infection has improved the response rate. In one study 14 days of therapy with telaprevir inhibited HCV replication in replicon based on a genotype 1b

HCV strain while introducing interferon and telaprevir in combination led to synergistic effect on HCV RNA suppression (Lin, Perni et al. 2006). In another similar study using genotype 1a replicon same response was seen (Perni, Almquist et al. 2006). Telaprevir is poorly effective in genotype 3 HCV and very few patients show some antiviral response (Foster, Hezode et al. 2011). In our experiments to see *in vitro* response to telaprevir in genotype 1 and 3 HCV, it is seen that sensitivity to telaprevir in the fusion assay correlates with the clinical response.

In summary, a novel fusion assay permitting *in vitro* replication of patient-derived HCV from diverse viral genotypes has been developed which could enable antiviral drug sensitivity testing. Genotype-specific responses to a novel antiviral drug - telaprevir have been demonstrated in this chapter and the assay shows promise for further development.

Chapter Five

Discussion

The aim of this thesis was to develop a functional model of HCV replication which examines virus derived from infected HCV patients. Hepatitis C is globally a major health issue and since its discovery scientists have faced difficulty with creating *in vitro* models. Despite many setbacks Interferon and ribavirin have been the standard of care (SOC) treatment for chronic HCV infection for almost a decade. However recently there have been developments in antiviral therapeutics. Novel direct acting antivirals (DAAs) have been instituted in the treatment of HCV and in future might replace present SOC therapy (reviewed in De Clercq 2012). Many other direct acting antiviral agents are being tested which target the protease NS3/4A, the protein NS5A, and the enzyme RNA dependent RNA polymerase 'NS5B'. Two of the DAAs already been introduced in the treatment of HCV are telaprevir and boceprevir (reviewed in De Clercq 2012).

The development of novel antiviral therapeutics can potentially be streamlined by using patient-derived model systems in which drugs can be tested prior to embarking upon clinical trials. Primary human hepatocyte cultures derived from hepatitis C patients have been used previously to study viral replication, life cycle, host interactions and to monitor response to antiviral medication (Rosenberg, Carpentier et al. 2010). However such models have been hampered by difficulties in maintaining primary human hepatocytes (PHHs) from infected individuals in culture for a long duration (reviewed in Raymond, Selliah et al. 2009) because of reduced cell viability and an inability to proliferate after isolation from *in vivo* environment mainly due loss of their differentiated phenotype (Bhatia, Balis et al. 1999, Wu, Robotham et al. 2012). Moreover, PHHs from uninfected individuals are

inefficiently infected by incubating in the sera of HCV infected patients (reviewed in Wu, Robotham et al. 2012). Few studies reported propagation of serum derived HCV into cultured PHHs (Rumin, Berthillon et al. 1999, Lazaro, Chang et al. 2007, Buck 2008, Molina, Castet et al. 2008) but the replication has been marginal and with no reproducibility (Ploss, Khetani et al. 2010). Nonetheless, PHHs withstood these barriers and were infected with HCVcc when cultivated with supporting cells in specialized micropatterned co-cultures. (Khetani and Bhatia 2008, Ploss, Khetani et al. 2010). However, these co-cultures need specialised substrates (culture-matrices) and expertise which are not yet widely available (Lindenbach 2010). Because of considerable difficulty in culturing PHHs researchers have attempted infection of immortalized hepatocytes and hepatoma cell lines (Ikeda, Kato et al. 1997, Ikeda, Sugiyama et al. 1998) but HCV titres were not very high (Ikeda, Sugiyama et al. 1998) and though suitable for evaluating antiviral drugs they are not sufficient to study viral life cycle.

As serum infected cell lines did not produce ample amount of HCV replication, studies were carried out to generate cell lines with full-length *in vitro* transcribed viral cRNA (Dash, Halim et al. 1997) or cDNA integrated into the chromosomes and thus expressing viral RNA. These clones were found to be infectious after intrahepatic inoculation in chimpanzees (Kolykhalov, Agapov et al. 1997, Yanagi, Purcell et al. 1997). A breakthrough in this field came with the invention of stable replication model of subgenomic HCV RNA and a bicistronic HCV replicon was cloned from patient with chronic hepatitis C (Lohmann, Korner et al. 1999). This system facilitated the study of different aspects of viral life cycle and the antiviral drug testing. It was however

noticed that replicon required several adaptive mutations in order to self replicate (Blight, Kolykhalov et al. 2000). This replicon system facilitated the study of viral life cycle and allowed antiviral drug testing. However the drawbacks of this system doesn't allow replication of the whole HCV making it difficult to interpret true response of antiviral drugs and true host-viral relationship cannot be assessed by this system (Guha, Lee et al. 2005). Moreover, adaptive mutations required for replication *in vitro* doesn't allow replication *in vivo* in chimpanzees which makes *in vitro* response to antiviral drugs less predictable (Bukh, Pietschmann et al. 2002).

It was observed that these conventional replicon systems were not able to produce infectious particles in supernatant (Arai, Suzuki et al. 2011) and this led to formation of a HCV infectious system by Wakita and his colleagues in which full length replicon was developed from genotype 2a infected Japanese patient with fulminant hepatitis and was called JFH-1. JFH-1 replicated efficiently and produced infectious virion in Huh-7 cell lines (Wakita, Pietschmann et al. 2005). Later structural region of J6 genotype 2a clone was introduced into JFH-1 which increased its infectivity (Lindenbach, Evans et al. 2005). Limitations of this system are that it contains HCV particles from genotype 2 strain which is less dominant genotype globally and was obtained from a fulminant hepatitis patient which is a rare event (reviewed in Yi, Villanueva et al. 2006). Infectious particles were also released by H77 a genotype 1a variant but infectivity of release particles is low compared to JFH-1 (Yi, Villanueva et al. 2006).

In order to create a model which could look at individual patient virus in more detail *in vitro* we used surplus tissue fragments of routine biopsy samples from patients with HCV to develop an *ex vivo* system to test antiviral compounds like telaprevir and NA808. Telaprevir is a first generation direct acting antiviral drug which inhibits viral protease NS3-4A and inhibits cleavage of nascent proteins involved in the assembly of nascent virions. In clinical trials telaprevir is more sensitive against genotype 1 strain of HCV (Reesink, Zeuzem et al. 2006). It is rapid acting and exerts its antiviral effect within 2 days of treatment however monotherapy is linked to emergence of resistance (Forestier, Reesink et al. 2007). NA808 is an experimental agent developed by Chughai Pharmaceutical Co. Ltd which could inhibit HCV replication in genotype 1 replicon system within 7 days according to experiments performed by Chughai Pharmaceutical Co. Ltd. Although its mechanism of action is not very clear it is thought to inhibit serine palmitoyltransferase enzyme which is a co-factor involved in sphingolipid synthesis pathway. As sphingolipid is involved in assembly of HCV its depletion can effect formation of infectious particles.

This thesis depends mainly on data gathered from real time qPCR reactions. The progress of real time qPCR and generation of amplicon can be monitored in real time. Apart from relative quantification we have used standard curve generation to assess copy numbers of HCV RNA. Due to exceedingly high sensitivity, despite being described as the *gold* standard, qPCR calls for appropriate standardization at every step along with optimal assay design and reliable data normalization. There is a chance of increased variability during qPCR due to assay design, PCR reagents, PCR

equipments and human error. It is essential to measure intra- and inter-assay variation and also to minimize variability. Hence, a well structured experimental design and use of appropriate controls can make real time PCR one of the most sensitive, efficient and reproducible methods of evaluating gene expression. In addition to qPCR we used indirect immunofluorescence to show viral NS5A protein co-localised with albumin which is produced specifically by Huh 7.5 cells.

Experiments to look at the effect of telaprevir on HCV RNA levels in intact liver biopsy fragments showed that there was a significant reduction in the mean HCV RNA in biopsies belonging to genotype 1 infected patients. However, the majority of biopsies showed only minute change in HCV RNA expression and the significant effects of telaprevir may have been dominated by the substantial changes seen in a few samples. Hence, the changes seen in most liver biopsies were modest and broad range of samples need to be analysed to examine the effects of the drug and derive a statistically significant result. Furthermore, effect of telaprevir on HCV RNA in liver biopsies on the whole did not show any significant change in genotype 2 and 3 infected patients. Thus, considering the effect of telaprevir on liver biopsies obtained from aggregated cohort of patients belonging to genotypes 1-3 show that the results are comparable to those derived from the clinical trials. Experiments with NA808 had limited sample size and short incubation period compared to previous experiments performed on replicon cells (containing genotype 1 strain of HCV) by 'Chugai Pharmaceuticals' in which effect was seen after incubation with NA808 for 7 days. Nonetheless, we observed that effect on HCV RNA after 24 hours incubation with NA808 was similar to that

obtained after 48 hours incubation. Genotype 3 strain infected biopsies showed response to NA808 both after 24 and 48 hours incubation. We could not however come to any definite conclusion about the effect of this drug on genotype 3 HCV RNA because of a very small sample size. Furthermore, It did not impart any inhibitory effect on HCV RNA from genotype 1 strain within 24 and 48 hours. It is not clear whether the lack of response in genotype 1 patients is due to lack of response to drug or due to longer action of NA808 which could not be seen in 48 hours. Nonetheless, it was found in later experiments on man that it did not cause any inhibitory effect at concentrations that were non toxic to humans and was withdrawn from further development.

Moreover, the need for multiple samples and statistical analysis detracts from the value of the assay – the data presented above indicate that it will be difficult to adapt this assay to determine the drug sensitivity of an individual patient. Poor sensitivity of the assay may be related, at least in part, to the differences in HCV RNA expression in different fragments taken from the same biopsy. Additionally, reduced viability of hepatic cells after isolation from *in vivo* environment may contribute to poor expression of HCV RNA and lack of sensitivity.

In summary, some of the drawbacks of liver biopsy model are high sample to sample variability and lack of homogeneity among different biopsy fragments, restricted availability of liver biopsies or resected liver tissues, limited duration of viability of the hepatic cells *in vitro*. Furthermore, differences in patients' clinical history, methods of isolation and host genetics contribute towards the lack of attainment of reproducible results.

The viability experiments showed that viability of liver biopsy fragments reduced considerably within 48 hours and extracting hepatic cells from the surrounding extracellular matrix led to further reduction in the viability of liver biopsy cells. The micro-patterning technique used by others groups may provide an optimal environment for maintaining hepatocytes in culture (Ploss, Khetani et al.2010, Khetani and Bhatia 2008). This micro-pattern co-culturing of hepatic cells with embryonic fibroblasts is used to provide a favourable microenvironment for adequate cell functioning and to achieve maximum benefits by controlling and modulating the degree of contact. This involves a miniaturized model with spherical islands of primary human hepatocytes co-cultivated with 3T3-J2 murine embryonic fibroblasts to induce heterotypic and homotypic synergy of cells. This interdependence is vital for preservation of the hepatocyte phenotype and function over prolong period as cells make polarized layers with optimized dimensions required for maintaining the viability and liver specific function. However, the system is technically very challenging and has not been reproduced outside the initiators laboratory. We used a simpler approach by growing hepatic cells with 3T3 embryonic fibroblasts in 1:4 ratio so that simple, easy to use culture conditions could be acquired that would maintain differentiated hepatocytes in culture for several days to allow studies on HCV replication and antiviral drug testing to be completed. In initial experiments comparing pure hepatic cells with co-cultured hepatic and 3T3 cells, HCV RNA was quantified to assess the viability of culture. A definite trend leveraging towards the side of co-culture was observed but antiviral drug testing showed considerable variation between duplicate samples and could not provide efficacy of this approach. Growing

hepatic cells with embryonic fibroblasts did not increase the value of the assay for antiviral drug testing in our liver biopsy studies. Thus this technique was abandoned.

Peripheral blood mononuclear cells harbour HCV and some studies have demonstrated HCV replication in these cells. However, there are speculations that there is low level replication in them (Cribier, Schmitt et al. 1995). We used polyethylene glycol mediated fused monocytes (from chronic HCV patients) and Huh 7.5 cells to produce a model for the study of HCV replication. Significant replication does not occur in Huh 7.5 cells incubated in patients' sera, this could be attributed to either a block in the uptake of virus or the lack of HCV receptors required for internalisation of the virus or a block in replication of HCV which could be subdued by delivery of large amounts of HCV by the fusion process to overcome host immune response. Moreover, other factors might play a part and further experimental work is needed to elucidate exact mechanism of monocyte/Huh 7.5 cells fusion induced HCV replication.

We determined optimum conditions which would provide maximum degree of fused monocytes and hepatocytic cells. Fusion experiments suggest that fusing monocytes from infected patients with Huh 7.5 cells leads to increased expression of HCV for the first 7 days after fusion and a decline in HCV RNA afterwards. HCV could not be stably expressed in Huh 7.5 cells for longer duration and establishment of an HCV infected cell line was not possible. Telaprevir was used to test validity of this approach because it is potent, fast acting and has shown genotype specific response in clinical trials (Foster, Hezode et al. 2011). Replication of HCV in fused cells could be inhibited by

telaprevir. In our experiments to look at telaprevir sensitivity in different genotypes it was demonstrated that response to telaprevir in the fusion assay correlates with that observed in clinical trials. Hence, HCV replication can be induced by fusion of infected patients' monocytes with Huh 7.5 cells and can be inhibited by antiviral compounds. To extend the applicability and practicality of the fusion assay we used monocytes from healthy patients to capture HCV from serum of chronically infected patients' sera and then fused these monocytes with Huh 7.5 cells and studied HCV replication further. As there is compartmental distribution of HCV quasispecies HCV from serum might vary compared to that harboured in monocytes (Roque-Afonso, Ducoulombier et al. 2005). Thus capturing HCV from serum might facilitate the direct examination of serum derived HCV after incorporating it into this technique. Replication was observed by indirect immunofluorescence microscopy in the capture-transfer model. Further analysis was carried out by Dr Morven Cunningham who used pre-stimulated monocytic cell line THP-1 to capture serum derived HCV. However, the issue with fusion approach is that the replication is marginal and the attempt to increase HCV replication in fused cells by interfering with IFN production (STAT1 degradation by PIV5) did not provide enhanced replication. As discussed in Chapter 1, Huh 7.5 cells are highly permissive to HCV replication in part due to defect in the RIG-1 molecule which is an important effector for the downstream production of IFN in response to viral RNA. The failure to enhance HCV replication in STAT1 degraded Huh 7.5 cell line could be that the Huh 7.5 cells are already deficit in IFN production and hence are highly permissive to HCV replication. This is due to the flaw in the RIG-1. STAT1 degradation in Huh 7.5 cells does

not increase HCV replication any further because IFN production is already defected in Huh 7.5 cells.

In short, our work demonstrates that HCV replication dies out after 7 days and thus a hybrid cell line containing replicating virus from HCV individuals for the purpose of long term drug sensitivity and resistance testing could not be developed. Nevertheless, our fusion technique has led to the formation of an ad hoc, interim model of patient derived HCV which can be used to study fast acting antivirals. Although this model is cell type dependent, it is potent and robust.

A large number of direct acting antivirals were developed using HCV subgenomic replicon system. However, determining sensitivity of new antiviral drugs to diverse viral genotypes often requires patient exposure which is not possible through the current HCV models. As the replication of patient-derived HCV in the fused cells could be inhibited by antivirals, this technique has a potential to be used as an identifier of patient-specific responses to antiviral compounds. HCV therapeutics involves the use of combination therapy to combat the development of resistance. Moreover, antiviral combination effective for one genotype or subtype differs from the other. For example, the combination of faldaprevir and non-nucleoside NS5B inhibitors is more effective against genotype 1b compared to genotype 1a (Kronenberger and Zeuzem 2008). Thus, accurately phenotyping the virus before commencing treatment is important.

We did not notice significant viral replication within monocytes. This could be due to either very low levels of HCV replication within monocytes or the virus

persists in a non-replicative form. Although the relationship between the therapeutic failure and the viral persistence in monocytes remains to be fully determined, the presence of replication competent virus in monocytes leads to the speculation that these cells may be acting as a 'Trojan horse' associated with the reappearance of HCV after therapeutic viral clearance..

This thesis has only looked at Huh 7.5 cell line which due to the defect in RIG-1 is highly permissive to HCV infection. Moreover, Huh 7.5 cells are hepatoma derived cells and might not represent PHHs. Moreover, the acquisition of PHHs is cumbersome and they lose their differentiated phenotype quickly on isolation from the *in vivo* environment. Additionally, their viability reduces rapidly *in vitro*, there is lot-to-lot variability amongst these cells and have low plating efficiency. Furthermore, stem cell technology has introduced a valuable alternative to Huh 7.5 cells by introducing the pluripotent stem cells, either obtained from embryonic source or induced by reprogramming factors like hESCs and iPSC respectively. These cells have ability to regenerate and have the potential to differentiate into hepatocyte like cells (Ghodsizadeh, Taei et al. 2010, Sullivan, Hay et al. 2010). Differentiated human hepatocyte-like cells (DHHs) display hepatic function, express hepatic markers and are permissive to HCVcc (Wu, Robotham et al. 2012, Roelandt, Vanhove et al. 2013). Moreover, the complete life cycle of HCV infection starting with viral entry and ending with the virion formation and secretion in culture medium has been observed in these cells. Both wild type and adaptive mutants were able to replicate in these cells indicating that the ability to support HCV infection does not depend upon particular mutations or isoforms (reviewed in Wu, Robotham et al. 2012).

In a nutshell, we have developed a novel fusion assay which permits replication of patient-derived HCV belonging to diverse range of genotypes *in vitro*. Furthermore, it could facilitate testing of patient sensitivities to antiviral drugs and genotype-specific response to novel antiviral drug telaprevir has been demonstrated. It can be used to predict the development of resistance in a chronic HCV patient and could be employed to distinguish poorly responsive viral subsets from responsive strains. The capture-fusion assay represents a promising new technique which may help identify the most appropriate treatment strategy for individual patients with chronic HCV infection. Further development is needed to convert this into a clinically beneficial assay for drug screening and determining resistance. It can be enhanced further to evaluate sensitivity of different patient isolates to telaprevir and to compare sensitivity in this model with deep sequencing and modified replicon. Moreover this technique could be used further in determining whether the transfer of other RNA or DNA viruses was possible by this approach or whether we could transfect monocytes with viral RNA or DNA and generate infection.

Appendices

Appendix 1

Knodel Histology activity index (HAI)

Periportal ± Bridging Necrosis	Score	Intralobular Degeneration and Focal Necrosis	Score	Portal Inflammation	Score	Fibrosis	Score
None	0	None	0	No Portal Inflammation	0	No Fibrosis	0
Mild piecemeal necrosis	1	Mild (acidophilic bodies, ballooning degeneration and/or scattered foci of hepatocellular necrosis in 1/3 of lobules or nodules)	1	Mild (sprinkling of inflammatory cells in <1/3 of portal tracts)	1	Fibrous portal expansion	1
Moderate piecemeal necrosis (involves <50% of the circumference of most portal tracts)	3	Moderate (involvement of 1/3-2/3 of lobules or nodules)	3	Moderate (increased inflammatory cells in 1/3-2/3 of portal tracts)	3	Bridging Fibrosis (portal-portal or portal-central linkage)	3
Marked piecemeal necrosis (involves >50% of the circumference of most portal tracts)	4	Marked (involvement of >2/3 of lobules or nodules)	4	Marked (dense packing of inflammatory cells in >2/3 of portal tracts)	4	Cirrhosis	4
Moderate piecemeal necrosis plus bridging necrosis	5						
Marked piecemeal necrosis plus bridging necrosis	6						
Multilobular necrosis	10						

Knodel 1981

Appendix 2

The Ishak Modification of the Knodell HAI:

Architectural Changes, Fibrosis and Cirrhosis

Change	Score
No fibrosis	0
Fibrous expansion of some portal areas, with or without short fibrous septa	1
Fibrous expansion of most portal areas, with or without short fibrous septa	2
Fibrous expansion of most portal areas, with occasional portal to portal bridging	3
Fibrous expansion of portal areas with marked bridging as well as portal-central	4
Marked bridging (portal to portal and/or portal-central) with occasional nodules (incomplete cirrhosis)	5
Cirrhosis, probable or definite	6
Maximum score	6

Ishak 1995

Appendix 3

The Ishak Modification of the Knodell HAI: Grading

Feature	Score
A. Periportal or periseptal interface hepatitis (piecemeal necrosis)	
Absent	0
Mild (focal, few portal areas)	1
Mild/moderate (focal, most portal areas)	2
Moderate (continuous around <50% of tracts or septa)	3
Severe (continuous around >50% of tracts or septa)	4
B. Confluent necrosis	
Absent	0
Focal confluent necrosis	1
Zone 3 necrosis in some areas	2
Zone 3 necrosis in most areas	3
Zone 3 necrosis + occasional portal-central bridging	4
Zone 3 necrosis + multiple portal-central bridging	5
Panacinar or multiacinar necrosis	6
C. Focal (spotty) lytic necrosis, apoptosis and focal inflammation	
Absent	0
One focus or less per 10X objective	1
Two to four foci per 10X objective	2
Five to ten foci per 10X objective	3
More than ten foci per 10X objective	4
D. Portal inflammation	
None	0
Mild, some or all portal areas	1
Moderate, some or all portal areas	2
Moderate/marked, all portal areas	3
Marked, all portal areas	4
Maximum score	18

Ishak 1995

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Publications Related to This Thesis

1. Patent: Method of Replicating HCV *in Vitro*
2. Development and validation of a 'capture-fusion' model to study drug sensitivity of patient-derived hepatitis C.

Patent

Method for replicating HCV in vitro

Method for replicating HCV in vitro

Publication number	WO2013064833 A1
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Inventors	Graham Foster, M.E. Cunningham, Alia Javaid
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Abstract

There is provided a method for replicating HCV virus in vitro which comprises the following steps: (i) fusing an HCV-infected white blood cell with a hepatocyte cell to produce a fused cell; and (ii) culturing the fused cell so that HCV replication may occur. There is also provided a fused cell capable of replicating HCV virus *in vitro* which is made by fusing an HCV-infected white blood cell with a hepatocyte cell, and uses of such a fused cell to screen for anti-HCV drugs and assessing patient responsiveness to therapy before treatment.

Claims (OCR text may contain errors)

1. A method for replicating HCV virus in vitro which comprises the following steps:
 - (i) Fusing an HCV-infected white blood cell with a hepatocyte cell to produce a fused cell; and
 - (ii) Culturing the fused cell so that HCV replication may occur.
2. A method according to claim 1, wherein the HCV-infected white blood cell is isolated from an HCV patient.
3. A method according to claim 2, wherein the HCV-infected white blood cell is made by infecting a white blood cell with HCV virus in vitro.
4. A method according to claim 3, wherein the white blood cell is derivable from a white blood cell line.
5. A method according to claim 3 or 4, wherein the white blood cell has been pre-treated with at least one pro-inflammatory reagent.
6. A method according to any preceding claim, wherein the white blood cell is a monocyte.
7. A method according to any preceding claim, in which white blood cell is infected with HCV virus having HCV genotype 2, 3, 4, 5 or 6.
8. A fused cell capable of replicating HCV virus in vitro which is made by fusing an HCV-infected white blood cell with a hepatocyte cell.
9. A method for making a fused cell according to claim 8, which comprises the step of fusing an HCV-infected white blood cell with a hepatocyte cell in vitro.
10. A method according to claim 9, wherein the HCV-infected white blood cell is derived from a HCV patient.
1. A method according to claim 9, wherein the HCV-infected white blood cell is made by infecting a white blood cell with HCV virus in vitro.
12. A method according to claim 1, which comprises the following steps:
 - (i) Culturing a white blood cell in vitro with serum from an HCV patient, so that the white blood cell is infected with HCV in the serum; and
 - (ii) Fusing the HCV-infected white blood cell with a hepatocyte.
13. The use of a fused cell according to claim 8 to assess the capacity of a test HCV treatment to reduce HCV replication.
14. A method for screening a test treatment which comprises the step of analysing the capacity of the test treatment to reduce HCV replication in a fused cell according to claim 7.

15. A method according to claim 14, wherein the test treatment is a test compound or composition. 16. A method according to claim 15, wherein the test compound or composition comprises IFN and/or ribavirin

17. A method according to claim 14, wherein the capacity of the test treatment is tested in a plurality of fused cells according to claim 8, each replicating a different strain of HCV virus, in order to analyse whether the effectiveness of the test compound or composition is HCV strain-specific.

18. A method for assessing the likelihood that a HCV patient will respond to a test HCV treatment, which comprises the step of analysing the capacity of the treatment to reduce HCV replication *in vitro* in a fused cell according to claim 8, wherein the fused cell is made by fusing a hepatocyte cell with a white blood cell which is either:

(i) Isolated from the HCV patient; or

(ii) A white blood cell which has been infected with HCV virus from the subject *in vitro*.

19. A method for selecting a treatment which comprises the step of assessing the responsiveness of an HCV patient to a test HCV treatment by a method according to claim 18 and selecting a treatment which reduces HCV replication in the fused cell *in vitro*.

20. A method for assessing the likelihood that a subject will relapse following treatment, which comprises the following steps: (i) fusing a white blood cell from the subject with a hepatocyte cell to produce a fused cell; and

(ii) Investigating whether HCV can be detected in the fused cell, wherein a subject is determined to be likely to relapse if HCV is detectable in the fused cell.

21. A method according to claim 20, wherein the presence of HCV is investigated by detecting the presence or absence of HCV RNA.

22. A method for investigating the progression of an HCV treatment which comprises the following steps:

(i) Periodically isolating a white blood cell from the subject;

(ii) Fusing the white blood cell with a hepatocyte cell to produce a fused cell; and

(iii) Investigating whether HCV can be detected in the fused cell.

23. A method for investigating the progression of an HCV treatment which comprises the following steps:

(i) Periodically isolating a serum from the subject;

(ii) Culturing a white blood cell in vitro with the serum, so that the white blood cell is infected with HCV in the serum; and

(iii) Fusing the HCV-infected white blood cell with a hepatocyte; and

(iv) Quantifying HCV RNA in the fused cell.

24. A method for determining when an HCV treatment may be stopped by investigating the progression of the treatment by a method according to claim 22 or 23 and determining that the treatment may be stopped when a white blood cell or a serum sample is isolated which produces a fused cell in which HCV is undetectable.

Detailed description of the patent is available at:

http://www.google.com/patents/WO2013064833A1?cl=en&utm_source=gb-gplus-sharePatent WO2013064833A1 - Method for replicating hcv in vitro

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Development and Validation of a “Capture-Fusion” Model to Study Drug Sensitivity of Patient-Derived Hepatitis C

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Emerging therapies for chronic hepatitis C viral (HCV) infection involve inhibition of viral enzymes with drug combinations. Natural, or treatment-induced, enzyme polymorphisms reduce efficacy. We developed a phenotyping assay to aid drug selection based on viral transfer from monocytes to hepatocytes. We studied HCV in monocytes from infected patients and developed a model in which patient-derived HCV is “captured” by the cell line THP-1 and replication assessed after fusion to hepatoma cells. We found that monocytes from HCV-infected patients harbor virus that replicates when cells are fused to hepatocytes. THP-1 cells incubated with infected sera capture HCV, which replicates when fused to hepatocytes. Inhibitable replication of all HCV genotypes was achieved (42 of 52 isolates). We measured sensitivity of telaprevir (TVR) and alisporivir (AVR) in different genotypes, and showed differences in 50% inhibitory concentration (IC₅₀) correlating with clinical response (TVR IC₅₀ for genotype (G)1 was 0.042 ± 0.003 vs. 0.117 ± 0.015 μ M for G3, whereas AVR IC₅₀ for G1 was 0.139 ± 0.013 vs. 0.044 ± 0.007 μ M for G3). We tested TVR-resistant viral isolates and identified changes in IC₅₀. One patient with a poor clinical response to TVR and wild-type viral sequence showed reduced TVR sensitivity in our assay. We studied samples from a 2-week TVR monotherapy study in which 5 of 8 patients with G3 HCV did not respond whereas 3 of 8 patients did. The “capture-fusion” assay correctly identified responders. **Conclusion:** The capture-fusion model represents a promising new technique that may help identify appropriate treatment strategies for patients with chronic HCV infection. (HEPATOLOGY 2015;61:1192-1204)

See Editorial on Page 1112

Therapy for patients infected with hepatitis C virus (HCV) is rapidly evolving, and interferon (IFN) and ribavirin (RBV) are being replaced by direct-acting antiviral (DAA) agents.¹ Drugs against the viral nonstructural protein 3 of HCV (NS3) protease (telaprevir [TVR] simeprevir,

and boceprevir) and nonstructural protein 5B of HCV (NS5B) polymerase (sofosbuvir; SOF) have been licensed, and new protease, nonstructural protein 5A of HCV (NS5A), and polymerase inhibitors are in clinical trials. In combination with IFN, most drugs increase the proportion of patients who respond. This has led to attempts to avoid IFN-related side effects by combining drugs of different classes in IFN-free regimens. Distinct viral strains respond differently, for

Abbreviations: Abs, antibodies; AVR, alisporivir; DAA, direct acting antiviral; DMEM, Dulbecco's modified Eagle medium; DMSO, dimethyl sulfoxide; FCS, fetal calf serum; G, genotype; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; JFH-1, Japanese fulminant hepatitis type 1; HCV, hepatitis C virus; IC₅₀, 50% inhibitory concentration; IF, immunofluorescence; IFN, interferon; IL, interleukin; mRNA, messenger RNA; NS3, nonstructural protein 3 of the hepatitis C virus; NS5A, nonstructural protein 5A of the hepatitis C virus; NS5B, nonstructural protein 5B of the hepatitis C virus; PBMCs, peripheral blood mononuclear cells; PEG, polyethylene glycol; Peg-IFN, pegylated IFN; PI3K/Pi4K, phosphatidylinositol 3- and 4-kinase; PMA, phorbol 12-myristate 13-acetate; PS, penicillin/streptomycin; RBV, ribavirin; RT-qPCR, reverse-transcription quantitative polymerase chain reaction; SOF, sofosbuvir; SR-B1, scavenger receptor class B type 1; SVR, sustained virological response; TVR, telaprevir; WT, wild type.

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example, genotype (G)1b strains respond well to protease inhibitors combined with non-nucleotide polymerase inhibitors or NS5A inhibitors,^{2,3} but G1a strains require additional drugs.⁴ Some G1a strains do respond to protease plus non-nucleoside drugs,⁵ but their characteristics are undefined. IFN-free SOF is effective for G2 HCV and has some efficacy in G1, but less so in G3.⁶

Development of DAAs was facilitated by *in vitro* HCV replication systems (the G1 replicon and replication-permissive G2 strain, Japanese fulminant hepatitis type 1 [JFH-1])^{7,8} that allowed high-throughput screening. Subgenomic replicons based around G3a and G4a have extended the range of genotypes that can be studied,^{9,10} and chimeric variants have been used to analyze resistance-associated variants. These systems are of limited value in analysis of a patient's viral sensitivity—defining drug sensitivity of a viral isolate requires subcloning each DAA target gene and insertion into a replicon. Difficulty in generating replicons with more than one modified locus precludes studies of multidrug combinations. In human immunodeficiency virus infection, viral phenotyping assays enabled selection of optimal regimens, although progress in genotypic resistance testing has reduced their value. For G1 HCV, some viral motifs associated with protease inhibitor treatment failure have been identified,¹¹ but for other genotypes there is no association between sequence and response,¹² indicating that, in the short term, viral phenotyping assays may be useful in identifying optimal regimens for less-responsive viral strains. Although recently presented data indicate that emerging drug regimens cure over 90% of patients with G1 HCV,^{13,14} the proportion of patients who fail may be higher in practice. To avoid development of “multidrug-resistant” HCV strains, careful selection of “second-line” regimens will be required. Identification of the most effective treatment regimens in this scenario would be facilitated by phenotyping assays.

Association of HCV with extrahepatic sites, such as peripheral blood mononuclear cells (PBMCs), is established, although extrahepatic viral replication remains controversial.^{15–20} Here, we describe a “capture-fusion” technique in which patient-derived HCV is “captured”

by monocytes and transferred to hepatocytes using cell fusion. This allows analysis of patient-derived virus from all genotypes and drug sensitivity and treatment response can be predicted. This new assay may be helpful in determining optimal drug combinations and be of particular value in patients who have failed “first-line” regimens.

Materials and Methods

Cells, Reagents, and Clinical Material. Huh7.5 cells (kindly provided by C.M. Rice, Rockefeller Institute, New York, NY) were maintained in Dulbecco's modified Eagle medium (DMEM) with glutamine, 10% fetal calf serum (FCS), and 1% penicillin/streptomycin (DMEM/10% FCS/PS). THP-1 cells²¹ were maintained in RPMI with glutamine, 10% FCS, and 1% PS.

PBMCs and sera were obtained from consenting patients with chronic HCV infection.

Stimulants/inhibitors used were phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich, Dorset, UK), IFN- γ (Invitrogen, Paisley, UK), and wortmannin (Sigma-Aldrich).

TVR and alisporivir (AVR) were kindly supplied by Janssen Virology (Baar, Switzerland) and Novartis Pharma AG (Basel, Switzerland), respectively. IFN- α -2a was from Cambridge Bioscience (Cambridge, UK) and RBV from Sigma-Aldrich. Ethical approval for the study was given by London-City Research Ethics Committee, and informed consent obtained from all patients. The study was conducted in accord with the Declaration of Helsinki.

Fusion of Patient Monocytes. PBMCs were separated from whole blood by centrifugation on Ficoll-Paque (GE Healthcare, Buckinghamshire, UK). The PBMC layer was washed twice before positive selection of CD14⁺ cells by magnetic separation (Miltey Bio-tec, Surrey, UK), according to manufacturer's instructions. For fusion optimization experiments, CD14⁺ cells were incubated with DiI and Huh7.5 cells with DiO (Life Technologies, Paisley, UK) before incubation with polyethylene glycol (PEG) 1500 (Roche Diagnostics, Burgess Hill, UK). Cells were fixed with 4% paraformaldehyde (PFA) and enumerated by flow

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Potential conflict of interest: Professor Foster consults, advises, is on the speakers' bureau for, and received grants from Gilead. He consults, advises, and is on the speakers' bureau for Boehringer Ingelheim, Bristol-Myers Squibb, Roche, Novartis, AbbVie, Merck, and Janssen. He received grants from Spring Bank.

cytometry (FACSCanto II; BD Biosciences, Oxford, UK).

For all subsequent fusion experiments, Huh7.5 cells and CD14⁺ cells (1:1) were incubated with PEG at 37°C for 2 minutes, then prewarmed medium (DMEM) added dropwise and the cells washed by centrifugation. Fused cells were seeded into six-well plates (5×10^5 cells/mL) and maintained at 37°C.

Stimulation and Infection of THP-1 Cells. THP-1 cells were seeded into six-well plates (10^6 cells/mL) and maintained for 18 hours, with or without IFN- γ (10 ng/mL) and PMA (200 ng/mL). Cells were washed thrice and medium replaced with RPMI/2% FCS and patient serum (1 HCV IU/cell). After incubation (37°C for 18–24 hours), supernatant was removed and cells washed. Adherent cells were removed by scraping and combined with Huh7.5 cells (1:1). Cell fusion was performed as described above. Fused cells were seeded into six-well plates (10^5 cells/mL) and maintained at 37°C with or without drugs for up to 7 days. In selected experiments, supernatants were pooled from non-drug-treated wells and concentrated by filtration through a 0.45- μ m filter, then 10 mL was layered on 4 mL of 20% sucrose. Supernatants were centrifuged (24,000g) for 2 hours and the pellet resuspended in 1 mL of RPMI. For capture-fusion experiments using concentrated supernatant, 1×10^6 prestimulated THP-1 cells were incubated with 1 mL of concentrated supernatant for 24 hours before fusion.

Drug Inhibition Assays. Fused cells were rested overnight before the addition of drugs. TVR and AVR were diluted from 20-mM stock solutions in dimethyl sulfoxide (DMSO). Each drug concentration was tested in quadruplicate. Drug dilution mix alone (RPMI/2% FCS/0.5% DMSO) was added to control wells. Media and drug were refreshed at day 3. Viral RNA was calculated as a percentage of that in untreated wells. Dose-response curves were constructed and used to estimate the 50% inhibitory concentration (IC₅₀) of drug using Prism 4.0 software (GraphPad Software, Inc., La Jolla, CA).

Cell Viability Assays. Cell viability was measured using the Cell Titer-Glo assay (Promega, Southampton, UK) and a FLUOstar Optima plate reader (BMG Labtech, Aylesbury, UK), according to the manufacturer's instructions.

Quantification of HCV RNA and Gene Expression. Total RNA was extracted with TRIzol (Invitrogen) and quantified using RiboGreen (Invitrogen), according to the manufacturer's instructions. Complementary DNA was synthesised using Moloney murine

leukemia virus reverse transcriptase (Promega) and diluted (1:10) for amplification by quantitative polymerase chain reaction (qPCR; QuantiTect SYBR Green PCR kit; Qiagen, Hilden, Germany). For primers and cycling conditions, see the Supporting Information. Target messenger RNA (mRNA) was normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) or β -actin expression, which were confirmed to be stably expressed in these experiments (GeNorm; Primer-Design, Southampton, UK).

For HCV copy number quantification, one-step reverse-transcription PCR (RT-qPCR) using the QuantiTect Virus Kit (Qiagen) and TaqMan Gene Expression Assay HCV primer and probe (Applied Biosystems, Paisley, UK) was performed. Serial dilutions of an RNA standard were included in each PCR run and results expressed relative to total sample RNA.

Fluorescence-Activated Cell-Sorting Analysis. Cells were incubated with primary antibody (Ab) or appropriate isotype control in 1% bovine serum albumin/10% FCS for 30 minutes. Alexa Fluor 488 secondary Abs were used to detect bound primary Ab and quantified on a FACSCanto II flow cytometer (BD Biosciences). Data were analyzed using FlowJo software (TreesStar, Ashland, OR).

Statistical Analysis. Data were tested for normality using the D'Agostino's and Pearson's test. Statistical analyses used the Student *t* test for parametric data or Mann-Whitney's U test for nonparametric data, with *P* < 0.05 considered significant.

Results

Identification of Replication-Competent HCV in Patient-Derived Monocytes by Fusion With Huh7.5 Cells. We examined CD14(+) monocytes from patients with chronic HCV infection (N = 4) and found low levels of HCV that did not increase during cell culture (7 days), implying a lack of viral replication (data not shown). We then fused patient-derived monocytes with Huh7.5 cells using PEG. Dye-labeled cells were used to determine optimal fusion conditions. Fusion of 40% of CD14(+) monocytes with Huh7.5 cells was achieved using optimized conditions (see Materials and Methods; Fig. 1A). Monocytes from patients infected with diverse viral genotypes were fused to Huh7.5 cells and maintained in culture for up to 21 days. HCV RNA increased progressively up to 7 days after fusion, but declined thereafter (Fig. 1B). In these preliminary experiments, HCV RNA was quantified relative to beta-actin expression. To confirm reliable detection of low-level HCV RNA and quantify

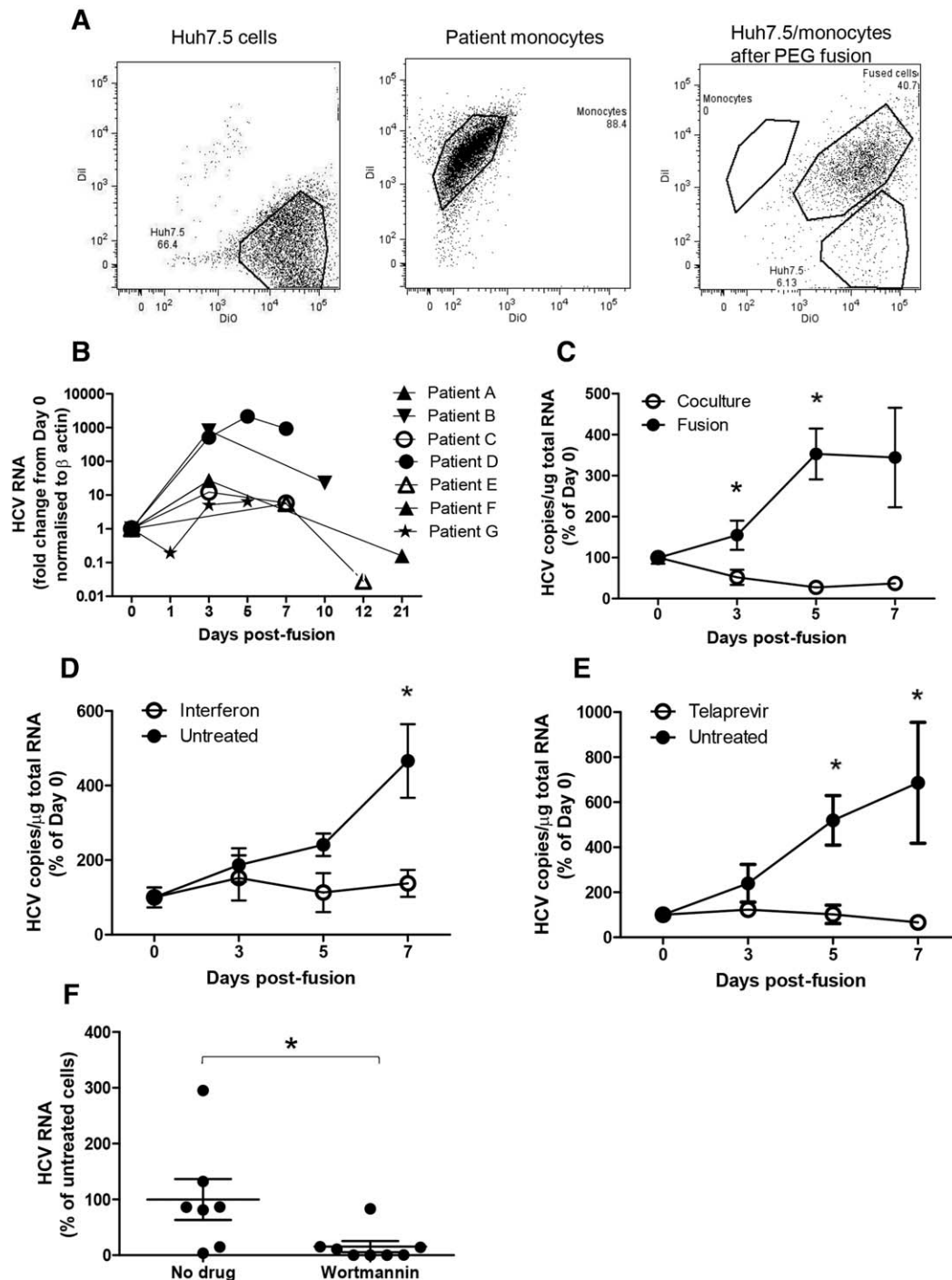


Fig. 1. Detectable HCV RNA increases after fusion of patient-derived monocytes with Huh7.5 cells, and this can be inhibited with antiviral drugs. (A) Fluorescence-activated cell-sorting plot showing PEG-mediated fusion of patient monocytes and Huh7.5 cells. Huh7.5 cells were stained with DiO and CD14(+) monocytes with DiI. After PEG fusion, the percentage of cells expressing both dyes was enumerated by flow cytometry. Figures refer to percentage of total cells. (B) monocytes from 7 patients with chronic HCV were fused with Huh7.5 and HCV RNA quantified at various times up to 21 days after fusion. Not all time points are available for all samples as a result of limited cell numbers. Results are expressed as fold change from unfused monocytes and normalized to beta-actin expression. (C) Mean \pm standard error of the mean (SEM) of five further experiments where monocytes from HCV patients were either fused or cocultured with Huh7.5 cells and HCV RNA quantified up to 7 days after fusion. $P = 0.038$ at 3 days and $P = 0.015$ at 5 days postfusion for the comparison between fused and unfused cells. (D) Mean \pm SEM of two further experiments where fused patient monocytes were cultured with or without 100 IU/mL of IFN- α -2a. $P = 0.031$ for the comparison between treated and untreated cells at day 7 postfusion. (E) Mean \pm SEM of three further experiments where fused G1 patient monocytes were cultured with or without TVR 0.5 μ M. $P = 0.0001$ at 5 days and $P = 0.0002$ at 7 days postfusion for the comparison between treated and untreated cells. (F) Mean \pm SEM of two further experiments where fused patient monocytes were cultured with or without the PI3K/PI4K inhibitor, wortmannin, 0.5 μ M for 5 days after fusion. $P = 0.014$ for the comparison between treated and untreated cells. (C-E) HCV copies/ μ g total RNA as a percentage of day 0 postfusion to normalize for differences in HCV-RNA yield between individual patient samples. (F) HCV copies/ μ g total RNA as a percentage of untreated cells at day 5 postfusion.

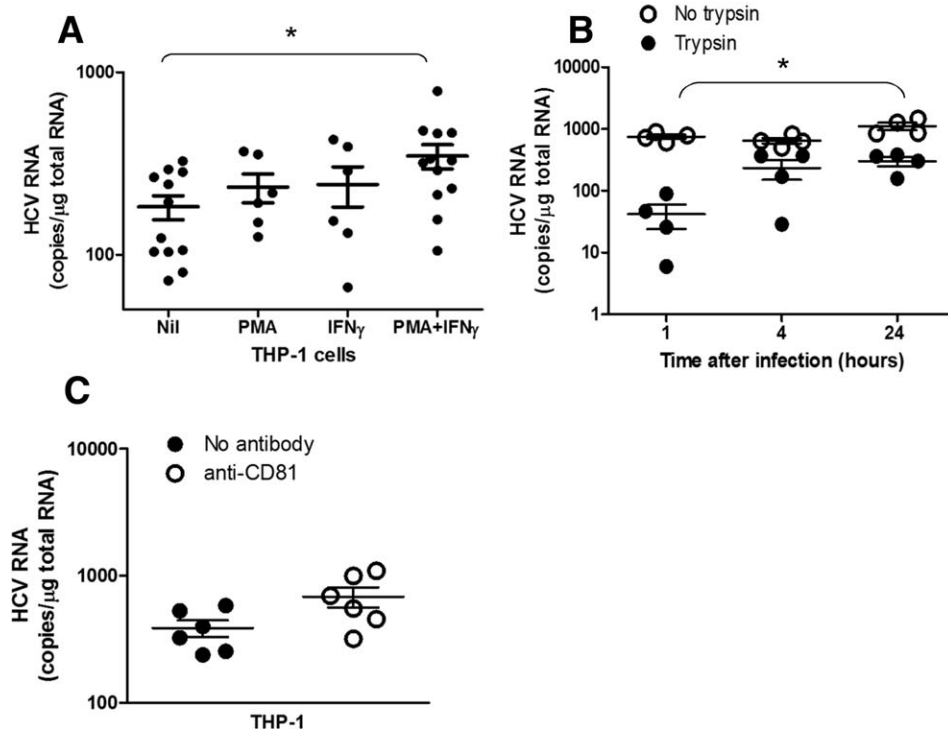


Fig. 2. Association of patient-derived HCV with THP-1 is enhanced by PMA/IFN- γ , but is independent of CD81. (A) THP-1 cells were cultured for 24 hours with PMA 200 ng/mL, IFN- γ 10 ng/mL, PMA 200 ng/mL and IFN- γ 10 ng/mL or no stimulants before incubation with patient serum. HCV RNA was measured 24 hours after infection. $P = 0.026$ for the comparison between unstimulated and PMA/IFN- γ -stimulated cells. (B) THP-1 cells prestimulated with PMA/IFN- γ were treated with trypsin at various times after incubation with patient sera. $P = 0.029$ for the comparison between trypsin treatment 0 and 24 hours postinfection. (C) PMA/IFN- γ -stimulated THP-1 cells were incubated in the presence or absence of anti-CD81 blocking Ab (5 μ g/mL) for 1 hour before infection with patient-derived HCV. HCV RNA was quantified after 24 hours. Graph shows mean \pm standard error of the mean of two independent experiments. $P = 0.09$ for the comparison between antibody treated and untreated cells.

HCV-RNA copy number, a one-step RT-qPCR assay was employed (see Materials and Methods) with a quantification range of 10 - 10^8 HCV copies per reaction (Supporting Fig. 1). Rather than logarithmic increases in HCV RNA observed in propagation of cell-culture HCV strains, we typically observed a 2- to 10-fold increase in viral RNA over 7 days after fusion, suggesting low-level replication (Fig. 1C). Treatment of fused cells with antiviral drugs abolished this increase (Fig. 1D,E). Treatment of fused cells with wortmannin, a phosphatidylinositol 3- and 4-kinase (PI3K/PI4K) inhibitor, also reduced replication after fusion (Fig. 1F). Contamination by laboratory HCV strains was excluded by viral sequencing (data not shown). Attempts to demonstrate HCV replication using less-sensitive techniques (western blotting and indirect immunofluorescence [IF]) were unsuccessful, most likely because of the low level of viral replication (data not shown). Together, these results indicate that viable HCV is associated with monocytes of patients with chronic HCV infection. Fusion of infected monocytes with Huh7.5 cells permits sufficient viral replication to be detected by a sensitive PCR assay.

Association of Patient-Derived HCV With THP-1 Cells Is Enhanced by PMA and IFN- γ , but Is Independent of CD81. Patient-derived HCV in fused cells can be inhibited with antiviral drugs, allowing identification of patient-specific responses to antiviral therapies. Given the limitations of working with patient monocytes, we examined whether cultured monocytes could be used to capture HCV from patient serum. After incubation of the monocytic cell line, THP-1, with patient serum, we found little association of HCV with THP-1 cells. Others report that PMA and IFN- γ pretreatment enhances HCV uptake into monocytic cell lines.²² Stimulation of THP-1 with PMA/IFN- γ enhanced the association of patient-derived HCV (Fig. 2A), and trypsin treatment of virus-loaded THP-1 cells after 4 hours reduced viral levels, indicating that virus was internalized between 1 and 4 hours after infection (Fig. 2B).

HCV entry into hepatocytes is dependent upon CD81, scavenger receptor class B type 1 (SR-B1), claudin, and occludin.²³ Neither mRNA nor cell-surface expression of CD81 or SR-B1 was enhanced by PMA/IFN- γ treatment of THP-1 (Supporting Fig.

2). Claudin-1 and occludin were not expressed by these cells. Blocking Abs to CD81 did not reduce HCV association with stimulated THP-1 (Fig. 2C), although JFH-1 entry into Huh7.5 cells was inhibited (Supporting Fig. 3). These data suggest that HCV association with THP-1 is independent of CD81 and other receptors are associated with HCV entry into hepatocytes.

Accumulation of Patient-Derived HCV Occurs After Capture Fusion. Accumulation of HCV RNA was observed after Huh7.5 fusion to PMA/IFN- γ -stimulated, HCV-exposed THP-1 cells, compared to HCV-exposed, PEG-treated Huh7.5, THP-1, or Huh7.5 alone (Fig. 3A). HCV-RNA accumulation after fusion was significantly greater when THP-1 were prestimulated, particularly when IFN- γ was added to PMA stimulation (Fig. 3B). To examine the potential antiviral effects of IFN- γ , we studied expression of the IFN-inducible gene MxA. MxA expression was not induced by cell fusion and was low in fusions with HCV-infected monocytes, even when the monocytes had been pretreated with PMA/IFN- γ , but could be up-regulated by subsequent treatment with IFN- α (Fig. 3C). Priming of Huh7.5 cells with IFN- α significantly reduced HCV replication after fusion (Fig. 3D). Thus, addition of IFN- γ increases uptake of virus by monocytes, but fusing IFN- γ -exposed cells does not lead to induction of antiviral genes in fused cells. However, direct stimulation of Huh7.5 cells with the more potent antiviral IFN, IFN- α , does reduce HCV replication. These data suggest PMA/IFN- γ treatment of THP-1 is important for HCV capture, but does not trigger induction of innate antiviral responses, although the cells are still able to induce antiviral genes.

To establish whether full replication cycles occurred in the fused cells with release of infectious virus, supernatants from capture-fusion experiments were collected, concentrated, and applied to Huh7.5 cells or to stimulated THP-1 cells in a fresh capture-fusion cycle. HCV RNA accumulated over time in fused cells and to a much lesser extent in Huh7.5 cells (Fig. 3E). These data suggest that HCV undergoes replicative cycles with release of infectious virions in the capture-fusion model.

HCV RNA Accumulation Occurs After Fusion in Isolates From All Viral Genotypes and Can Be Inhibited by Antiviral Drugs. Fifty-two viral isolates of genotypes 1-6 were tested in this capture-fusion assay and HCV RNA could be quantified (>10 copies per reaction) after fusion in 42 (81%). Median viral yield was 63 copies/ μ g total RNA (range, 14-354)

equivalent to median 19 HCV copies per fused cell (range, 4-106). Of the remaining 10 samples, seven were archival (3-7 years old) and may have degraded during storage. Three samples produced detectable HCV RNA below the quantification limit in repeated experiments. HCV-RNA yield varied between samples, but did not relate to viral load or disease activity (Supporting Fig. 4), and was consistent in repeated experiments for a given sample (Supporting Fig. 5), suggesting that replicative fitness in this model is influenced by intrinsic viral characteristics. TVR exposure after fusion caused a dose-dependent reduction in HCV RNA in isolates from all genotypes except G3, known to be poorly TVR sensitive (Fig. 3F). Experiments with alternative inhibitors confirmed that sensitivity of G3 HCV could also be studied (see below).

Drug Sensitivity of Patient-Derived HCV in the Capture-Fusion Model Reflects Genotypic Patterns of Response to Antiviral Drugs. We observed that G3 HCV was poorly inhibited by TVR in the capture-fusion assay (Fig. 3F). TVR inhibits G1, but has little effect against G3 HCV.²⁴⁻²⁶ Conversely, the cyclophilin inhibitor, AVR, is more efficacious against G2 and G3 than G1 and G4.²⁷ THP-1 cells exposed to sera from G1- and G3-infected donors were fused with Huh7.5, then treated with TVR or AVR for 5 days before quantification of HCV RNA. Dose-response curves were constructed and used to calculate an IC₅₀. Figure 4 shows pooled results from 14 donors, normalized to untreated cells. Dose-response curves were constructed for all isolates which reached quantifiable levels (Supporting Fig. 6 shows absolute values for representative patients). Drug-induced cytotoxicity was excluded by measuring cell viability after drug exposure. TVR had no effect on cell survival. The minor effects of doses of AVR >0.1 μ M were insufficient to account for the reduction in HCV RNA (Supporting Fig. 7). G1 samples were significantly more sensitive to TVR and less sensitive to AVR than G3 (Fig. 4A-D). Individual TVR and AVR IC₅₀s for each sample are shown in Fig. 4E,F. G2 isolates were sensitive to TVR and AVR, whereas G4 strains showed a similar AVR response to G1 (in keeping with clinical trial data). Some G4 isolates were poorly sensitive to TVR, consistent with clinical trial findings.²⁸ However, other G4 isolates were inhibited by TVR in the assay, suggesting differing TVR susceptibility amongst G4 strains (Supporting Fig. 8).

Drug Sensitivity in the Capture-Fusion Assay Reflects Phenotypic Treatment Response. To explore whether clinical TVR resistance could be detected in the capture-fusion assay, pretreatment and post-

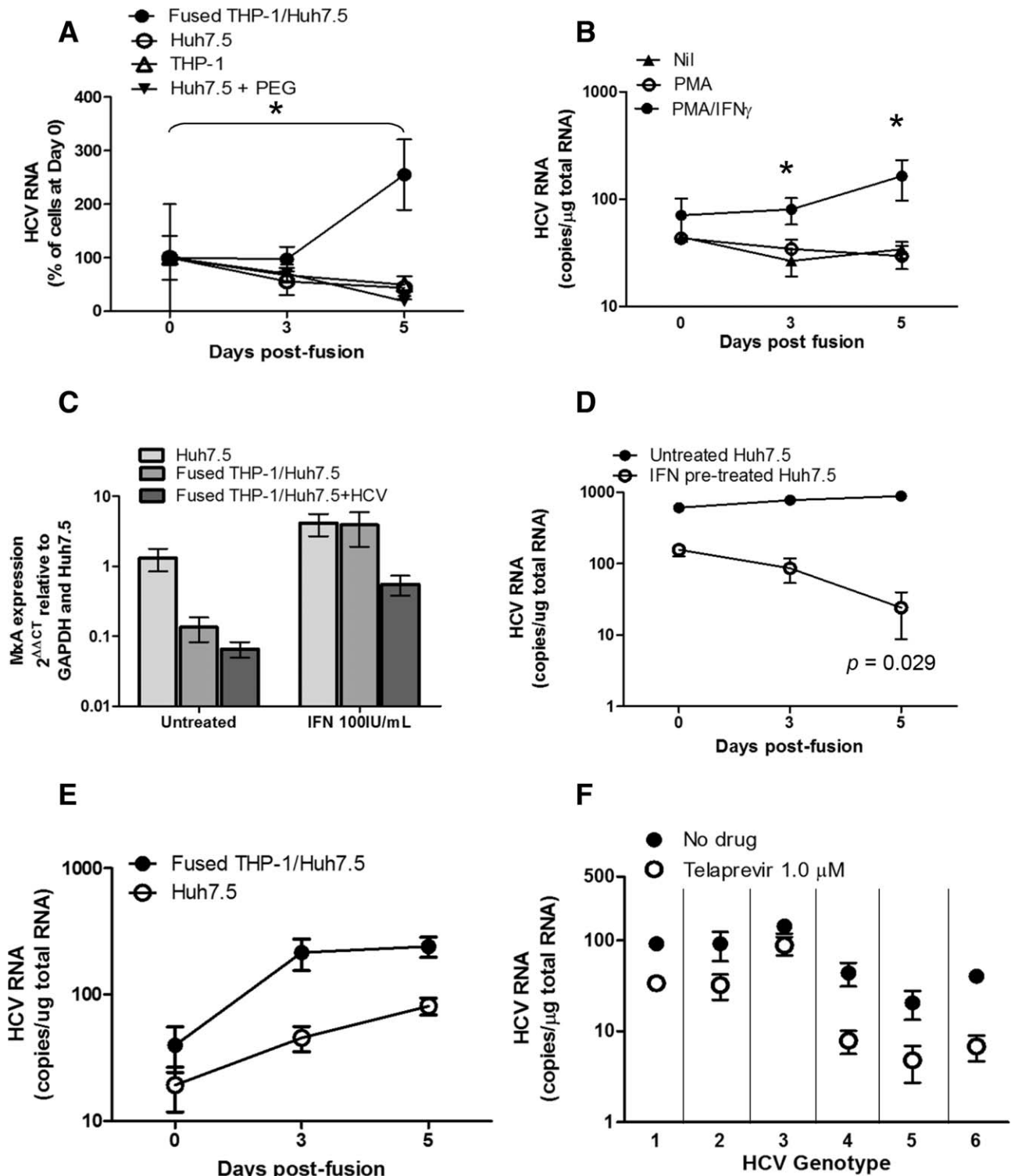


Fig. 3. Replication of patient-derived HCV occurs after capture by prestimulated THP-1 and fusion with Huh7.5 cells and can be inhibited by telaprevir. A. Mean \pm standard error of the mean (SEM) of two independent experiments where HCV RNA was quantified from PMA/IFN- γ -stimulated, HCV-exposed THP-1 fused with Huh7.5 cells, from HCV-exposed THP-1 and Huh7.5 cells without fusion, and from HCV-exposed Huh7.5 cells treated with PEG (fusion protocol). $P = 0.019$ for the comparison between HCV RNA in fused THP-1/Huh7.5 at days and 5 postfusion. Results are shown as percentage of HCV RNA in cells at day 0. (B) Representative of two independent experiments where THP-1 cells were cultured with PMA 200 ng/mL, PMA 200 ng/mL and IFN- γ 10 ng/mL or no stimulants for 24 hours before infection with patient-derived HCV and fusion with Huh7.5 cells. HCV RNA was quantified up to 5 days after fusion. $P = 0.005$ and $P = 0.015$ for the comparison between unstimulated and PMA/IFN- γ -stimulated THP-1 at days 3 and 5 postfusion, respectively. (C) PMA/IFN- γ -treated THP-1 fused with Huh7.5 (labeled fused THP-1/Huh7.5), PMA/IFN- γ -treated, HCV-exposed THP-1 fused with Huh7.5 (labeled fused THP-1/Huh7.5 + HCV), or unfused Huh7.5 (labeled Huh7.5) were cultured for 5 days in the presence or absence of IFN- α -2a (100 IU/mL). MxA mRNA was quantified by PCR and expressed relative to GAPDH expression and uninfected, unfused, untreated Huh7.5. Results are shown on a logarithmic scale. (D) Huh7.5 cells were pre-treated with IFN- α -2a (100 IU/mL) before fusion with prestimulated, HCV-infected THP-1. HCV RNA was quantified up to 5 days after fusion. (E) Mean \pm SEM of two independent experiments in which supernatant from capture-fusion experiments was applied to PMA/IFN- γ -stimulated THP-1, which were then fused with Huh7.5 in a further round of capture-fusion, or naïve Huh7.5 cells. HCV RNA was quantified up to 5 days after fusion. (F) HCV RNA 5 days after fusion in capture-fusion experiments, with or without TVR 1.0 μ M using sera from donors infected with diverse HCV genotypes. Each point represents mean HCV RNA \pm SEM from at least four biological replicates from a representative patient sample.

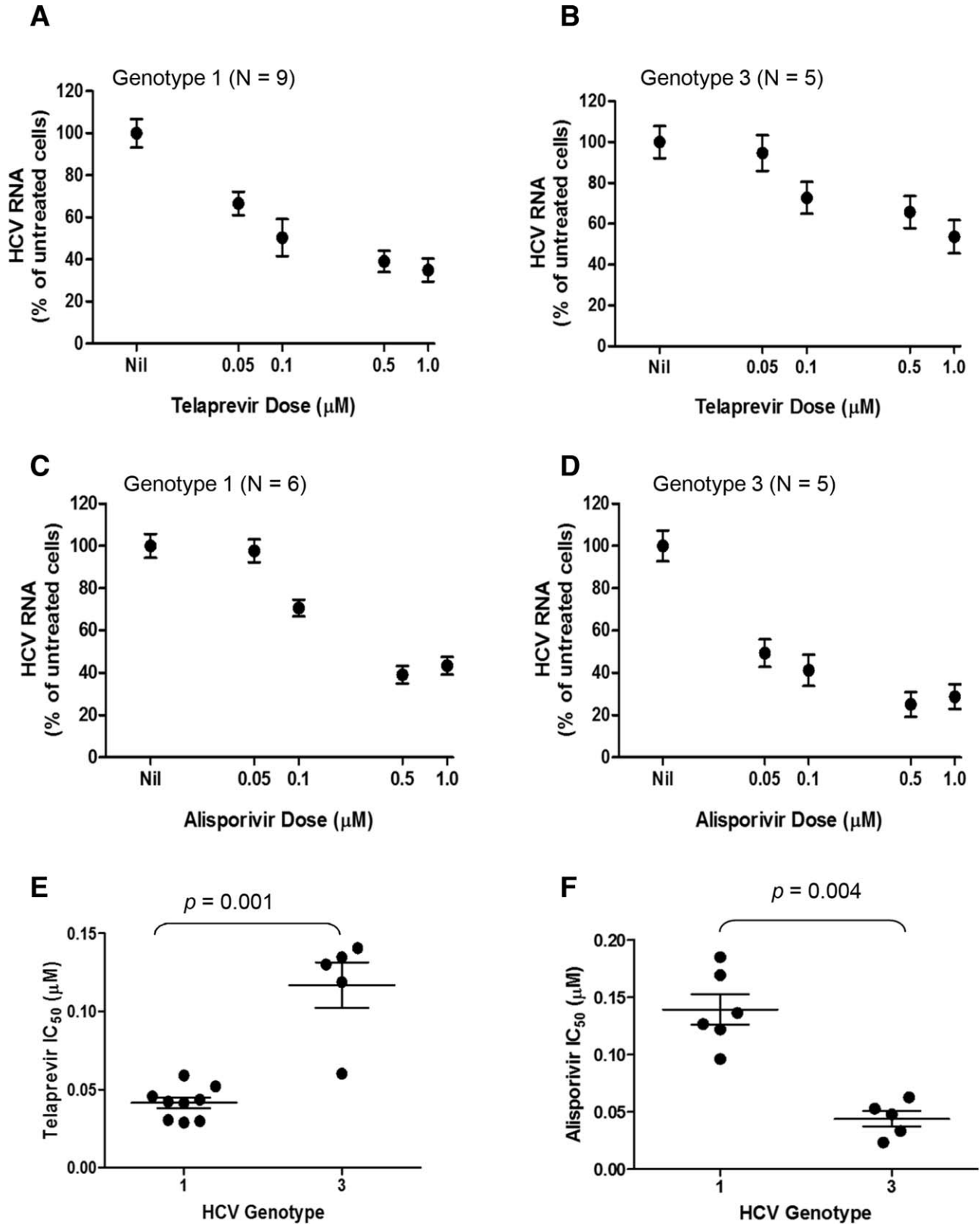


Fig. 4. Sensitivity of patient-derived HCV to antiviral drugs can be assayed in the capture-fusion model. HCV derived from serum from 9 G1 and 5 G3 donors was treated with varying concentrations of TVR or AVR in capture-fusion assays and HCV RNA measured after 5 days. G1 patient-derived HCV was more sensitive to TVR than G3 (A and B), but less sensitive to AVR (C and D). Comparison of individual IC₅₀ values showed TVR IC₅₀ was 0.042 ± 0.003 for G1 samples versus 0.117 ± 0.015 μM for G3; $P = 0.001$ (E). Conversely, AVR IC₅₀ was 0.139 ± 0.013 for G1 samples versus 0.044 ± 0.007 μM for G3; $P = 0.004$ (F). Graphs show mean \pm standard error of the mean.

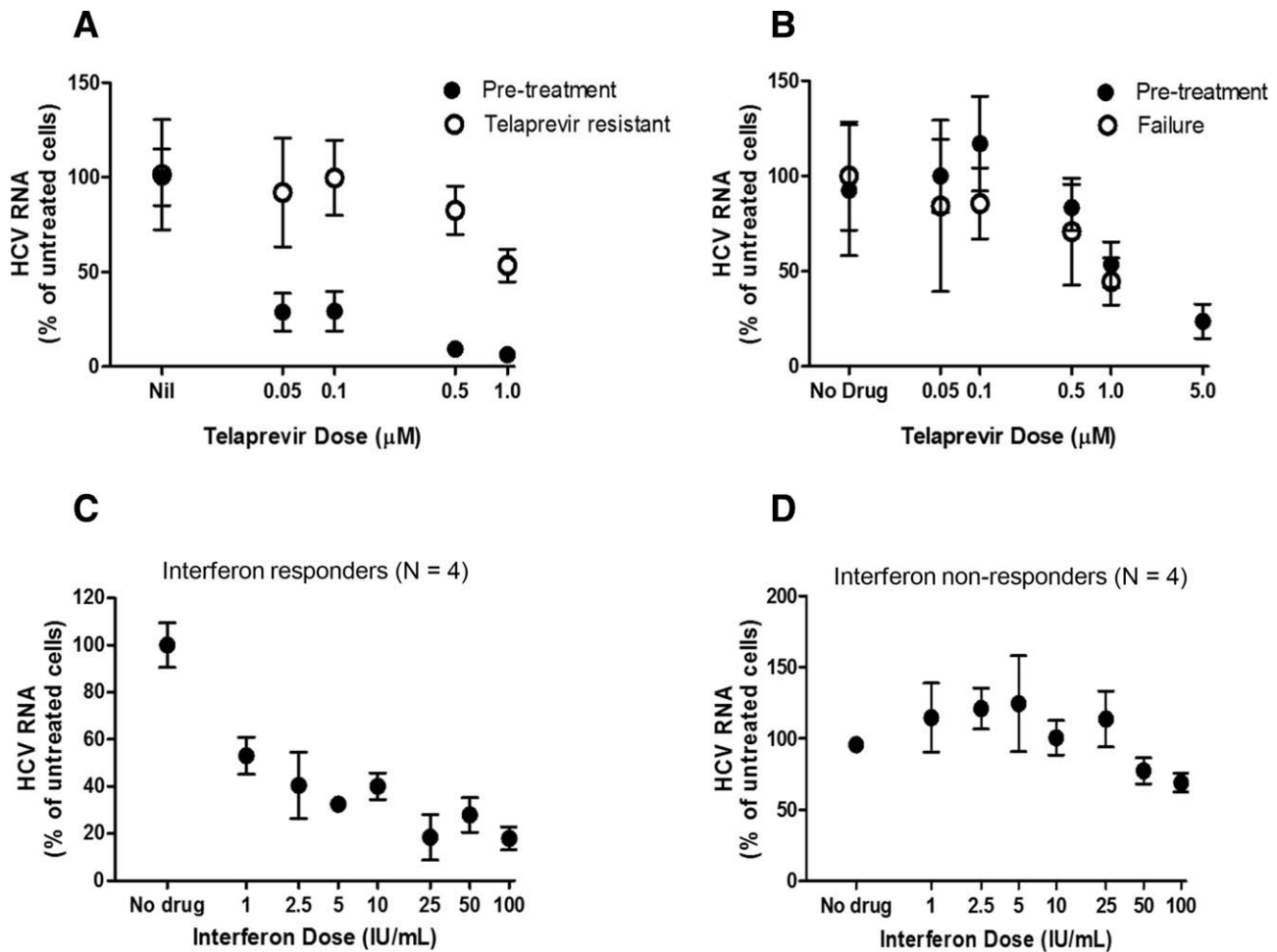


Fig. 5. Phenotypic and genotypic drug resistance correlates with drug sensitivity in the capture-fusion assay. Pretreatment and postbreakthrough sera from 2 patients who failed treatment with TVR, Peg-IFN, and RBV demonstrated loss of TVR sensitivity in the capture-fusion assay after acquisition of genotypic and clinical TVR resistance (A). Pre- and post-treatment serum from a patient with little clinical response to TVR, Peg-IFN, and RBV showed poor TVR sensitivity both pretreatment and after treatment failure (B). Using archived pretreatment sera from patients who did or did not respond to Peg-IFN and RBV treatment, HCV strains from patients who responded to treatment (C) were more sensitive to IFN in the capture-fusion assay than viral strains from patients who did not respond (D). Graphs show mean \pm standard error of the mean.

breakthrough sera were obtained from 2 patients with G1 HCV who initially responded to TVR, pegylated IFN (Peg-IFN) and RBV, but experienced virological breakthrough by treatment week 12. Sequencing of the first ~200 amino acids of viral NS3 to 1% frequency by pyrosequencing found wild-type (WT) virus at baseline and TVR resistance-associated mutations V36M/R155K at breakthrough in the majority of sequences. TVR sensitivity pre- and postbreakthrough assessed in the capture-fusion assay demonstrated a loss of sensitivity in the resistant strains (Fig. 5A). Pre- and post-treatment serum was obtained from 1 further patient with G1 HCV with a poor clinical response to TVR, Peg-IFN, and RBV (circa 1 log₁₀ reduction in viral load at week 4). No TVR resistance-associated mutations were identified pretreatment (sequencing to 1% frequency), although V36M/R155K mutations

were present at treatment failure. This strain demonstrated poor TVR sensitivity in the capture-fusion assay both before and after exposure (Fig. 5B). Pretreatment sera from 2 further patients with G1 HCV and a poor clinical response to TVR, Peg-IFN, and RBV also demonstrated poor TVR sensitivity in the capture-fusion assay (data not shown). This suggests that phenotypic sensitivity to TVR may be more predictive of response than genotypic sensitivity.

IFN sensitivity may be a determinant of acquisition of resistance to direct-acting antiviral drugs. Whether host or viral factors dominate IFN sensitivity is unclear. Archived pretreatment sera from 4 patients who responded to Peg-IFN and RBV (HCV RNA undetectable at treatment week 4) and 4 patients with a null response (<2 log₁₀ drop in viral load at treatment week 12) were used to examine IFN sensitivity

in the capture-fusion assay. Pretreatment HCV from patients who responded to IFN was more sensitive to IFN in the capture-fusion assay than virus from patients with a null response (Fig. 5C,D). Patient viral loads and viral replication achieved in the assay did not differ significantly between the groups (data not shown).

In contrast to G1, the predominant mode of treatment failure in G3 HCV infection is relapse. Relapse rates are higher in patients who receive suboptimal RBV dosing.^{29,30} Archived pretreatment sera from 10 G3 patients with cirrhosis treated with Peg-IFN and RBV, 4 with sustained virological response (SVR), and 6 who relapsed were used to study pretreatment IFN and RBV sensitivity. No difference in IFN sensitivity was observed, but pretreatment HCV from patients with SVR was more sensitive to RBV in the assay than virus from relapsers (Fig. 6).

Sensitivity to TVR in the Capture-Fusion Assay Correlates With Clinical Response. Although TVR was poorly effective in G3 HCV, a subgroup of patients (~30%) did respond.²⁶ To establish whether the capture-fusion assay could identify TVR-sensitive G3 HCV, pretreatment, blinded sera were obtained from 8 patients with G3 HCV who had received TVR monotherapy in a clinical trial.²⁶ TVR sensitivity of each sample in the capture-fusion assay correlated with clinical response, whereas a biochemical phenotyping assay did not¹² (Fig. 7). Three samples with the greatest TVR sensitivity in the capture-fusion assay corresponded to patients who responded clinically to TVR. These results indicate that the assay may predict patient responses to TVR.

Discussion

We have developed a capture-fusion technique that allows drug sensitivity of over 80% of HCV viral strains to be assessed. We found that resistance to TVR can be reliably determined, clinical sensitivity to cyclophilin and protease inhibitors can be identified, and there is a strong correlation between TVR sensitivity in our assay and clinical response in patients with G3 HCV, unlike conventional phenotyping assays. Cell-fusion techniques have been employed to investigate extrahepatic HCV replication,³¹ but this is the first report using cell fusion to facilitate replication of patient-derived HCV strains.

Emerging therapies for chronic HCV infection involve combinations of DAAs. Different combinations have differential effects on different viral strains, for example, faldaprevir with a non-nucleoside NS5B inhibitor cures most patients with G1b HCV, but only

43% of patients with G1a.² Accurate phenotyping of infecting virus before antiviral therapy may be valuable in determining appropriate drug combinations in future therapeutic regimens. Although the number of drug combinations tested in our assay is relatively small, we have no reason to expect that the assay will not identify viral sensitivity to a wide range of different therapies.

We investigated compounds with clinical trial data that differ in different genotypes. We found a close correlation between activity in patients and our assay, suggesting that the capture-fusion model may be useful in assessing sensitivity to DAAs. For the protease inhibitor, TVR, our assay accurately identifies viruses with known resistant variants, and, of interest, we identified a single patient with WT protease sequence and a poor therapeutic response to TVR whose virus was phenotypically resistant to TVR. These data suggest that novel resistant variants to TVR may exist and lie outside the classical resistance-associated regions. Further work will be required to delineate this region and identify the frequency of these variants.

The clinical response to Peg-IFN and RBV therapy for HCV is dominated by host (interleukin [IL]28B)^{32,33} and viral genotype. In an assessment of viral IFN sensitivity in 8 patients with diverse responses, we noted reduced sensitivity in patients who showed no clinical response. The archival samples analyzed could not be correlated with IL28B genotype, and insufficient samples were available to assess RBV sensitivity. Further studies will be needed to investigate the complex interactions between host IL28B genotype, viral IFN sensitivity, and clinical outcome. We noted a reduced response to RBV in patients with G3 HCV who relapsed, when compared to patients who achieved SVR, suggesting that RBV sensitivity may be an important determinant of response in this genotype. Further studies, including viral sequencing and phenotyping in chimeric replicons, will be required to confirm this observation and are underway.

It has long been recognized that monocytes from patients with HCV contain viral fragments, but it is unclear whether these cells contain replicating virus. We find that fusing monocytes from patients with HCV to replication-permissive hepatocytes allows viral replication in chimeric cells, indicating that monocytes contain viable HCV. The role of sequestered virus in viral persistence and the emerging immune response remains to be determined.

Interestingly, given the antiviral action of IFN- γ , we found that IFN- γ prestimulation of THP-1 was required, in addition to PMA, to achieve viral replication after fusion of HCV-exposed THP-1 with

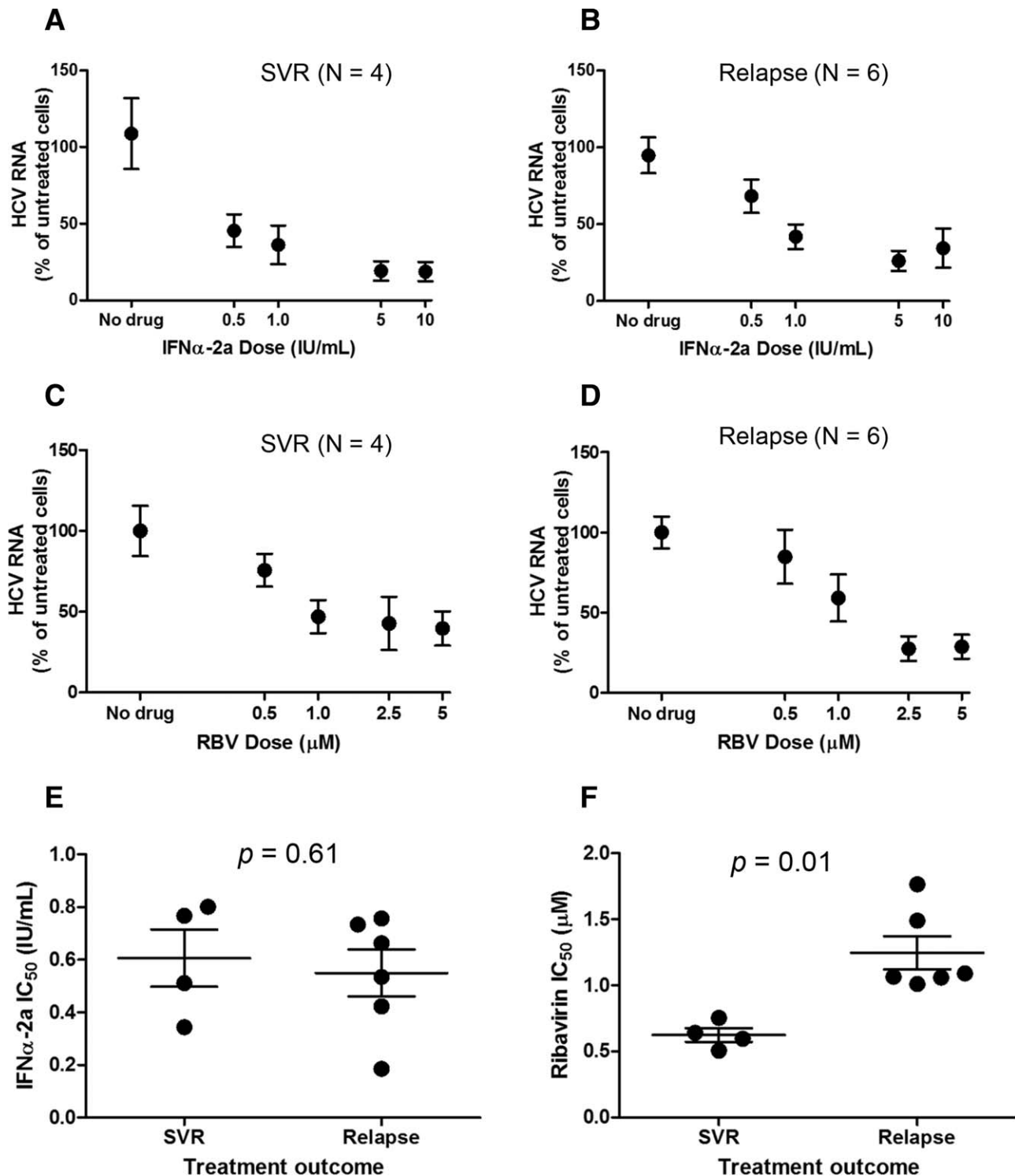


Fig. 6. Pre-treatment RBV sensitivity in the capture-fusion assay correlates with treatment response in G3 patients with cirrhosis treated with IFN/RBV. Using archived pretreatment sera from 10 G3 patients with cirrhosis who relapsed or achieved SVR after Peg-IFN and RBV treatment, no difference in IFN sensitivity was observed according to treatment outcome (A, B, and E). HCV strains from patients who achieved SVR were more sensitive to RBV in the capture-fusion assay than viral strains from patients who relapsed (C, D, and F). Graphs show mean \pm standard error of the mean.

Huh7.5. Others have observed Fc receptor up-regulation after PMA/IFN- γ treatment of monocyte cell lines, with enhanced uptake of HCV immune

complexes.²² Certainly, uptake of HCV into THP-1 cells appears independent of the classical HCV entry receptors, CD81, SR-B1, claudin, and occludin.

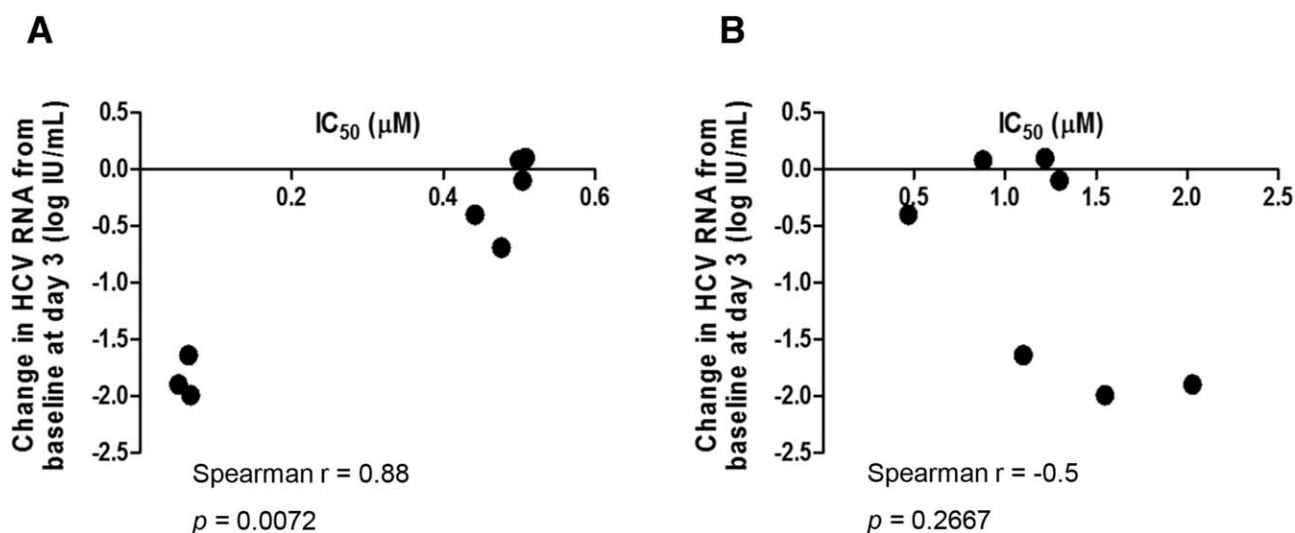


Fig. 7. Correlation between pretreatment TVR sensitivity and response to TVR in 8 patients with G3 HCV. Clinical response to TVR monotherapy is shown on the y-axes and could be predicted by the capture-fusion assay (A), but not by a biochemical phenotyping assay in which the viral NS3 was subcloned and enzymatic activity assessed in the presence and absence of TVR using a fluorescence resonance energy transfer cleavage assay¹² (B).

Significant replication of HCV does not occur in THP-1 cells, suggesting that fusion with Huh7.5 may provide access to permissive factors absent in THP-1. These may include the phosphoinositide kinases, PI3K or PI4K, given that the PI3K/PI4K inhibitor, wortmannin, reduced replication after fusion. Delivery of HCV to Huh7.5 cells by fusion bypassed MxA induction, despite retention of IFN-signaling pathways. This suggests that one mechanism underlying the capture-fusion process is delivery of virus to hepatocytes without an intracellular innate immune response. However, other factors are likely to play a role and elucidating the mechanisms underlying HCV replication after monocyte-hepatocyte fusion requires further work.

The assay described depends upon detection of low-level HCV RNA by PCR. Alternate techniques to detect HCV proteins directly (e.g., western blotting and IF) were attempted, but the level of viral protein was too low to allow detection (western blotting) or was masked by high levels of nonspecific background staining in fused cells (IF). PCR is a well-validated technique to detect viral replication, and the robust responses observed in the assay support the value of this approach.

In summary, we have developed a capture-fusion assay that permits replication of patient-derived HCV of all viral genotypes *in vitro* and enables testing of sensitivity to antiviral drugs. Genotype-specific responses to novel antiviral drugs and detection of resistant viral strains have been demonstrated. IFN-responsive or poorly responsive viral strains can be distinguished, and the assay can identify the subgroup of

patients with G3 HCV who respond to TVR. The capture-fusion assay represents a promising new technique that may help identify the most appropriate treatment strategy for patients with chronic HCV.

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Supporting Information

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